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declare as follows:

1. That I am well acquainted with both the English and Japanese languages, and

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   Japanese Patent Application No. 2004-213743
   Entitled: "Dopaminergic neuronal progenitor marker Lrp4/Corin"
   Filed on July 22, 2004

September 27, 2010
Date

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【Name of Document】 APPLICATION FOR PATENT

【Identification Number】 E1-A0404

【Filing Date】 July 22, 2004

【Addressee】 Esq. Commissioner of the Patent Office

【International Patent Classification】 C12Q 01/068

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【Payment】

【Registration Number】 041092

【Amount】 16000 yen

【List of Attached Documents】

【Name of Document】 Claim 1

【Name of Document】 Specification 1

【Name of Document】 Drawings 1

【Name of Document】 Abstract 1

[Document Name] Claims

[Claim 1]

A dopaminergic neuron proliferative progenitor cell marker polynucleotide probe comprising a sequence selected from the following nucleotide sequences (1) to (5):

(1) a nucleotide sequence complementary to a nucleotide sequence of SEQ ID NO: 1 or 2;

(2) a nucleotide sequence complementary to a nucleotide sequence encoding an amino acid sequence of SEQ ID NO: 3 or 4;

(3) a nucleotide sequence complementary to a nucleotide sequence encoding a sequence lacking a transmembrane domain in an amino acid sequence of SEQ ID NO: 3 or 4;

(4) a nucleotide sequence that hybridizes under stringent conditions with a polynucleotide consisting of a nucleotide sequence of SEQ ID NO: 1 or 2; and,

(5) a nucleotide sequence comprising at least 15 contiguous nucleotides selected from sequences of (1) to (4).

[Claim 2]

A method for selecting a dopaminergic neuron proliferative progenitor cell, wherein the method comprises the step of contacting the polynucleotide of claim 1 with a cell sample thought to comprise a dopaminergic neuron proliferative progenitor cell.

[Claim 3]

A method for selecting a dopaminergic neuron lineage cell, wherein the method comprises the steps of:

(1) selecting a dopaminergic neuron proliferative progenitor cell using the method of claim 2 for selecting the dopaminergic neuron proliferative progenitor cell;

(2) culturing the proliferative progenitor cell selected in step (1); and

(3) screening the cells cultured in step (2) by using a marker for a postmitotic dopaminergic neuron.

[Claim 4]

A dopaminergic neuron proliferative progenitor cell prior to cell division arrest, which is selected by the method of claim 2.

[Claim 5]

A method for isolating a dopaminergic neuron progenitor cell-specific gene and a gene specific for each maturation stage from the progenitor cell to a dopaminergic neuron, wherein the method comprises the step of detecting and isolating a gene specifically expressed in the proliferative progenitor cell of claim 4 or a cell which is differentiated, induced, or proliferated from the proliferative progenitor cell.

[Claim 6]

A method of screening for a compound which regulates proliferation and/or differentiation of a

dopaminergic neuron lineage cell using maturation as an index, wherein the method comprises the steps of: contacting a test substance with the proliferative progenitor cell of claim 4 or a cell which is differentiated, induced, or proliferated from the proliferative progenitor cell; and detecting a change of the proliferative progenitor cell or the progenitor cell caused by the contact.

[Claim 7]

An antibody against a polypeptide selected from the following (1) to (6):

(1) a polypeptide encoded by a nucleotide sequence of SEQ ID NO: 1 or 2;

(2) a polypeptide comprising an amino acid sequence of SEQ ID NO: 3 or 4;

(3) a polypeptide comprising an amino acid sequence lacking a transmembrane domain in an amino acid sequence of SEQ ID NO: 3 or 4;

(4) a polypeptide comprising an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in an amino acid sequence of SEQ ID NO: 3 or 4;

(5) a polypeptide encoded by a nucleotide sequence that hybridizes under stringent conditions with a sequence complementary to a nucleotide sequence of SEQ ID NO: 1 or 2; and,

(6) a polypeptide that is a fragment of a polypeptide of (1) to (5) comprising at least eight amino acid residues.

[Claim 8]

The antibody of claim 7, which is produced by the hybridoma (FERM AP-20120 or FERM AP-20121).

[Claim 9]

A dopaminergic neuron progenitor cell marker antibody, which comprises the antibody of claim 7 or 8.

[Claim 10]

A method for selecting a dopaminergic neuron progenitor cell, wherein the method comprises the step of contacting the antibody of any one of claims 7 to 9 with a cell sample thought to comprise a dopaminergic neuron progenitor cell.

[Claim 11]

A method for selecting a dopaminergic neuron lineage cell, wherein the method comprises the steps of:

(1) selecting a dopaminergic neuron progenitor cell using the method for selecting a dopaminergic neuron progenitor cell of claim 10;

(2) culturing the progenitor cell selected in step (1); and

(3) screening the progenitor cells cultured in step (2) by using a marker for a postmitotic dopaminergic neuron.

[Claim 12]

A dopaminergic neuron progenitor cell, which is selected by the method of claim 10.

[Claim 13]

A method for isolating a dopaminergic neuron progenitor cell-specific gene and a gene specific for each maturation stage from the progenitor cell to a dopaminergic neuron, wherein the method comprises the step of detecting and isolating a gene specifically expressed in the progenitor cell of claim 12 or a cell which is differentiated, induced, or proliferated from the progenitor cell.

[Claim 14]

A method of screening for a compound which regulates proliferation and/or differentiation of a dopaminergic neuron lineage cell using maturation as an index, wherein the method comprises the steps of: contacting a test substance with the progenitor cell of claim 12 or a cell which is differentiated, induced, or proliferated from the progenitor cell; and detecting a differentiated or proliferated progenitor cell caused by the contact.

[Document Name] Specification

[Title of the Invention] Dopaminergic neuronal progenitor marker Lrp4/Corin

[Technical Field]

[0001]

Lrp4, encoding a transmembrane protein, was identified as a dopaminergic neuron progenitor cell-specific gene. It was confirmed that Lrp4 mRNA and Lrp4 protein specifically express in dopaminergic neuron proliferative progenitor cells and in dopaminergic neuron progenitor cells containing cells prior to and after cell division arrest, respectively. Thus, the present invention relates to polynucleotide probes and antibodies for detecting dopaminergic neuron progenitor cell marker Lrp4/Corin that enable efficient isolation of dopaminergic neuron progenitor cells, which can be used for transplantation therapy of neurodegenerative disorders such as Parkinson's disease (PD), and methods for selecting progenitor cells by using them.

[Background Art]

[0002]

The dopamine system is an extremely important system for essential motor regulation, hormone secretion regulation, emotion regulation, and such in the mammalian brain. Thus, abnormalities in dopaminergic neural transmission cause various neural disorders. For example, Parkinson's disease (PD) is a neurodegenerative disease of the extrapyramidal system that occurs due to specific degeneration of dopaminergic neurons in the substantia nigra of the midbrain (Harrison's Principles of Internal Medicine, Vol. 2, 23rd edition, Isselbacher *et al.*, ed., McGraw-Hill Inc., NY (1994), pp. 2275-7). As a primary therapeutic method for Parkinson's disease, oral administration of L-DOPA (3,4-dihydroxyphenylalanine) is performed to compensate for the decrease in the amount of dopamine produced; however, the duration of the effect is known to be unsatisfactory.

[0003]

More recently, a therapeutic method for Parkinson's disease was attempted in which the midbrain ventral regions of 6 to 9-week old aborted fetuses containing dopaminergic neuron progenitor cells are transplanted to compensate for the loss of dopaminergic neurons (Patent Document 1; and Non-Patent Documents 1 to 6). However, in addition to cell supply and ethical issues (Rosenstain (1995) Exp. Neurol. 33: 106; Turner *et al.* (1993) Neurosurg. 33: 1031-7), this method is currently under criticism for various other problems, including risk of infection and contamination, immunological rejection of transplants (Lopez-Lozano *et al.* (1997) Transp. Proc. 29: 977-980; Widner and Brudin (1988) Brain Res. Rev. 13: 287-324), and low survival rates due to the primary dependence of fetal tissues on lipid metabolism rather than glycolysis (Rosenstein (1995) Exp. Neurol. 33: 106).

[0004]

In order to resolve the ethical issues and shortage of supply, method has been proposed that use, for example, porcine cortex, stria, and midbrain cells (for example, see Patent Documents 2 to 4). In this method, a complex procedure that involves altering cell surface antigens (MHC class I antigens) is required to suppress rejection. A method involving local immunosuppression by simultaneously transplanting Sertoli cells has been proposed as a method for eliminating transplant rejection (Patent Documents 5 and 6; and Non-Patent Document 7). It is possible to obtain transplant cells from relatives that have matching MHCs, bone marrow from other individuals, bone marrow banks, or umbilical cord-blood banks. However, if it were possible to use the patient's own cells, the problem of rejection reactions could be overcome without any laborious procedures or trouble.

[0005]

Therefore, as transplant materials, the use of dopaminergic neurons differentiated *in vitro* from non-neural cells such as embryonic stem (ES) cells and bone marrow interstitial cells, instead of cells from aborted fetuses, is considered to be promising. In fact, functional dopaminergic neurons were reported to have been formed by transplanting ES cells to lesion stria of a rat Parkinson's disease model (Non-Patent Document 8). It is believed that the importance of regenerative therapy from ES cells or the patient's own nerve stem cells will increase in the future.

[0006]

In treating damaged nerve tissue, it is necessary to reconstruct brain function, and in order to form a suitable link with surrounding cells (network formation), it is necessary to transplant immature cells, cells capable of differentiating into neurons *in vivo*. In the transplanting of neuron progenitor cells, in addition to the aforementioned problem regarding supply, there is also the possibility that progenitor cells will differentiate into groups of heterogeneous cells. In treating Parkinson's disease, it is necessary to selectively transplant the catecholamine-containing neurons that produce dopamine. Examples of transplant cells that have previously been proposed for use in the treatment of Parkinson's disease include striatum (Non-Patent Documents 3 and 9), immortalized cell lines derived from human fetal neurons (Patent Documents 7 to 9), human postmitotic neurons derived from NT2Z cells (Patent Document 10), primordial neuron cells (Patent Document 11), cells and bone marrow stromal cells transfected with exogenous genes so as to produce catecholamines such as dopamines (Patent Documents 12 and 13), and genetically engineered ES cells (Non-Patent Document 8). Additionally, the use of dopaminergic neurons formed by contacting nerve progenitor cells derived from fetal midbrain tissue with FGF-8 and Shh (Patent Document 14), and of tyrosine hydroxylase-expressing cells obtained by treating NT2 nerve cells with

retinoic acid (Patent Document 15) has been proposed. However, none of these contain only dopaminergic neurons or cells that differentiate into dopaminergic cells.

[0007]

A method has been proposed for selectively concentrating and isolating dopaminergic neurons from undifferentiated cell populations. In this method, a reporter gene that expresses a fluorescent protein is introduced into each cell of a cell population, under the control of a gene promoter/enhancer such as the tyrosine hydroxylase (TH) expressed in dopaminergic neurons, and then cells emitting fluorescence are isolated. The dopaminergic neurons are visualized in their viable state, then concentrated, isolated, and identified (Patent Document 17). This method requires the complicated step of introducing an exogenous gene, and further, the presence of a reporter gene poses problems of toxicity and immunogenicity when used in conjunction with gene therapy.

[0008]

[Patent Document 1] US Patent No. 5690927

[Patent Document 2] Japanese Patent Application Kohyo Publication No. (JP-A) H10-508487 (unexamined Japanese national phase publication corresponding to a non-Japanese international publication)

[Patent Document 3] JP-A (Kohyo) H10-508488

[Patent Document 4] JP-A (Kohyo) H10-509034

[Patent Document 5] JP-A (Kohyo) H11-509170

[Patent Document 6] JP-A (Kohyo) H11-501818

[Patent Document 7] JP-A (Kohyo) H08-509215

[Patent Document 8] JP-A (Kohyo) H11-506930

[Patent Document 9] JP-A (Kohyo) 2002-522070

[Patent Document 10] JP-A (Kohyo) H09-5050554

[Patent Document 11] JP-A (Kohyo) H11-509729

[Patent Document 12] JP-A (Kohyo) 2002-504503

[Patent Document 13] JP-A (Kohyo) 2002-513545

[Patent Document 14] US Patent No. 6277820

[Patent Document 15] International Publication WO 00/06700

[Patent Document 16] Japanese Patent Application Kokai Publication No. (JP-A) 2002-51775 (unexamined, published Japanese patent application)

[Non-Patent Document 1] Spencer *et al.* (1992) N. Engl. J. Med. 327: 1541-8

[Non-Patent Document 2] Freed *et al.* (1992) N. Engl. J. Med. 327: 1549-55

[Non-Patent Document 3] Widner *et al.* (1992) N. Engl. J. Med. 327: 1556-63

[Non-Patent Document 4] Kordower *et al.* (1995) N. Engl. J. Med. 332: 1118-24

[Non-Patent Document 5] Defer *et al.* (1996) Brain 119: 41-50
[Non-Patent Document 6] Lopez-Lozano *et al.* (1997) Transp. Proc. 29: 977-80
[Non-Patent Document 7] Selawry and Cameron (1993) Cell Transplant 2: 123-9
[Non-Patent Document 8] Kim *et al.* (2002) Nature 418: 50-56
[Non-Patent Document 9] Lindvall *et al.* (1989) Arch. Neurol. 46: 615-31

[Disclosure of the Invention]

[Problems to be Solved by the Invention]

[0009]

One of the major problems in PD transplantation therapy at the moment is that both *in vitro* differentiated dopaminergic neuron progenitor cells and midbrain ventral region of aborted fetuses are mixtures of a myriad cell types. When considering safety in neural circuit formation, it is preferable to use isolated cells that comprise only the cell type of interest. Furthermore, when considering the risk of tumorigenesis, it is believed better to use isolated postmitotic neurons. Moreover, when considering the survival of cells at their transplantation site in the brain, and their ability to properly form a network, it is expected that therapeutic effects can be further improved by isolating progenitor cells at as early a stage as possible.

[Means for Solving the Problems]

[0010]

In order to isolate genes specific to dopaminergic neuron progenitor cells, a gene specifically expressed in the most ventral region of the E12.5 murine midbrain containing dopaminergic neurons was identified using a modification ("Method for Homogenizing the Amounts of DNA Fragments and Subtraction Method", Japanese Patent Application No. 2001-184757 (filing date 2001/6/19) of the subtraction method (N-RDA: Representational Difference Analysis; RDA (Listsyn N.A. (1995) Trends Genet. 11: 303-7) by additionally dividing the ventral region into two regions in the dorsoventral direction. As a result, the present inventors successfully isolated a novel gene 65B13 as one of transiently-expressed genes in postmitotic neuron progenitor cells (International Publication Pamphlet WO 2004/038018). Furthermore, one of the isolated fragments is a cDNA fragment encoding Lrp4/Corin. Lrp4 encoded a type II transmembrane protein (Fig. 1).

[0011]

Lrp4 mRNA is specifically expressed in a ventral midline region in the midbrain. The areas match the areas where dopaminergic neuron proliferative progenitor cells are present. Furthermore, when Lrp4 expression is compared to that of TH, which is a marker for dopamine neurons, their signals are in identical dorsoventral positions, but they do not overlap (Figs. 4 and 6). This indicated that Lrp4 mRNA is not expressed in these progenitor

cells, which had stopped division and migrated to the neural tube outer layer. Therefore, by using Lrp4 mRNA as an index, it is possible to specifically detect/select dopaminergic neuron proliferative progenitor cells.

[0012]

Thus, the present invention provides dopaminergic neuron proliferative progenitor cell marker polynucleotide probes capable of specifically detecting Lrp4 mRNA, and methods for selecting dopaminergic neuron proliferative progenitor cells utilizing these probes. Furthermore, the present invention relates to dopaminergic neuron proliferative progenitor cells prior to cell division arrest, selected by using such nucleotide probes; as well as methods for isolating dopaminergic neuron proliferative progenitor cell-specific genes and genes specific for each maturation stage from progenitor cell to dopaminergic neuron, utilizing the proliferative progenitor cells; and methods of screening for compounds which induce differentiation or proliferation of the progenitor cells, using maturation as an index. It is also possible to culture the proliferative progenitor cells selected by using the nucleotide probes of the present invention, and to obtain dopaminergic neuron lineage cells comprising the postmitotic dopaminergic neuron progenitor cells. The term "dopaminergic neuron lineage cells" used herein refers to dopaminergic neuron proliferative progenitor cells prior to cell division arrest, postmitotic dopaminergic neuron progenitor cells, and/or dopaminergic neurons. The dopaminergic neuron lineage cells can also be utilized for the methods for isolating genes specific to each stage of maturation to dopaminergic neuron, and the methods of screening for compounds which induce the differentiation or proliferation of progenitor cells, using maturation as an index. Therefore, the present invention relates to methods for obtaining the dopaminergic neuron lineage cells by culturing the dopaminergic neuron proliferative progenitor cells selected by using the nucleotide probes of the present invention; cells obtained in this way; methods for isolating genes specific to each stage of maturation to dopaminergic neurons that use the cells; and methods of screening for compounds which induce the differentiation or proliferation of the cells using maturation as an index.

[0013]

Furthermore, the present inventors produced an anti-Lrp4 antibody, and examined Lrp4 protein expression. First, by analyzing its expression in tissues (Fig. 8), the Lrp4 protein was confirmed to be expressed in the same way as Lrp4 mRNA. In this experiment, Lrp4 protein signals were also detected in TH-expressing areas. However, since the proliferative progenitor cells extend processes toward the outer layer of the neural tube, this signal could not be determined to be caused by detecting proteins on the processes or the TH-expressing cells also expressed the Lrp4 protein. Next, by using the anti-Lrp4 antibody, it was confirmed that Lrp4 protein was expressed on the cell surface by FACS analysis. ES

cells in which Lrp4 mRNA expression was confirmed were prepared by inducing the differentiation *in vitro* (SDIA method) as samples. As a result, it was confirmed that the Lrp4 protein was certainly expressed on the surface of the cells (Fig. 9). Such proteins expressed on the cell surface are particularly preferable to use as separation markers because living cells can be selected (see Fig. 15).

[0014]

Next, Lrp4-positive cells were separated from cells induced by SDIA method and from mouse fetal ventral midbrain cells by a cell sorter using the anti-Lrp4 antibody. Gene expression in the separated cells was analyzed by the RT-PCR method. As a result, expression of the proliferative progenitor cell marker Nestin was observed. In addition, cells expressing MAP2, which is a postmitotic marker, were also revealed to be included (Fig. 10). TH and Nurr1, which are markers for postmitotic dopaminergic neurons, were expressed at higher levels in an Lrp4-positive cell population than in an Lrp4-negative cell population. Therefore, unlike in the case of using Lrp4 mRNA as the index, when cells are selected using antibodies by using Lrp4 protein as an index, it is possible to isolate the dopaminergic neuron progenitor cells, including postmitotic dopaminergic neuron progenitor cells.

[0015]

Accordingly, the present invention provides antibodies which specifically detect the Lrp4 protein and methods that utilize the antibodies to select dopaminergic neuron progenitor cells. Furthermore, the present invention relates to dopaminergic neuron progenitor cells selected by using such antibodies; as well as methods that use the progenitor cells to isolate dopaminergic neuron progenitor cell-specific genes and genes specific for each maturation stage from progenitor cell to dopaminergic neuron; and methods of screening for compounds which induce the differentiation or proliferation of the progenitor cells using maturation as an index. It is also possible to obtain dopaminergic neuron lineage cells at other differentiation stages by culturing the progenitor cells selected using the antibodies of the present invention. Such cells can also be utilized in the methods for isolating genes specific to each stage of maturation to dopaminergic neuron, and the methods of screening for compounds which induce the differentiation or proliferation of the progenitor cell using maturation as an index. Accordingly, the present invention also relates to methods for obtaining dopaminergic neuron lineage cells by culturing dopaminergic neuron progenitor cells selected using the antibodies of the present invention; cells obtained using the methods; methods that use the cells to isolate genes specific to each stage of maturation to dopaminergic neurons; and methods of screening for compounds which induce the differentiation or proliferation of the cells using maturation as an index.

[Effects of the Invention]

[0016]

Lrp4 expresses in heart from fetal life to adulthood , and is type II transmembrane protease that is speculated to cleave atrial natriuretic peptide (ANP), which is a blood pressure control hormone. ANP precursor is expressed as pro-ANP. After secreted to outside the cell, pro-ANP is cleaved by Lrp4 on the surface of the cell membrane to be an active ANP. There have been no previous reports of genes encoding membrane proteins specifically expressed in dopaminergic neuron progenitor cells. Antibodies to Lrp4 protein expressed on the cell membrane surface are believed to be extremely effective in isolating Lrp4-expressing cells. For example, pure dopaminergic neuron cells can be obtained by isolating Lrp4-expressing cells from the ventral midbrain region or cultured cells containing dopaminergic neuron cells differentiated *in vitro*, using anti-Lrp4 antibodies (Fig. 15).

[0017]

Moreover, the progenitor cells can also be transplanted directly, or after having been grown *in vitro*. The progenitor cells of the present invention also have the potential to differentiate and mature at the optimum region in the brain, as well as the potential to additionally grow *in vivo*, and can be expected to demonstrate long-term therapeutic effects. In addition, if Lrp4-expressing cells are transplanted after having differentiated and matured *in vitro*, they can be expected to demonstrate therapeutic effects, even if for some reason they do not differentiate into dopaminergic neurons *in vivo*. In consideration of the risks of tumorigenesis and such, an even higher degree of safety can be expected if cells that have been isolated using a postmitotic neuron marker such as 65B13 after differentiating Lrp4-expressing cells grown *in vitro* are transplanted. The use of Lrp4-expressing cells for transplantation therapy after being isolated, regardless of the method, enables a high degree of safety since only the cell type of interest is isolated. In addition, since the earliest progenitor cells can be used, high therapeutic efficacy can be expected in terms of survival rate, network formation ability, and such. Further, even if the best therapeutic effects cannot be achieved by these early progenitor cells immediately after isolation, since progenitor cells isolated using markers of the present invention can mature *in vitro* by culturing or such, materials in the optimum stage of differentiation can be prepared (Fig. 6).

[0018]

On the other hand, obtaining pure dopaminergic neuron progenitor cells are also useful in the search for therapeutic targets for Parkinson's disease, such as for use in the isolation of genes specific to dopaminergic neurons. In particular, obtaining proliferative progenitor cells are useful for research on the maturation process of dopaminergic neurons, screening systems using maturation as an index, drug screening in which progenitor cells are grown *in vitro* or *in vivo*, screening for drugs that induce differentiation of progenitor cells *in*

vivo (*in vivo* regenerative therapy drugs), and the like.

[Best Mode for Carrying Out the Invention]

[0019]

<Marker Polynucleotide Probes>

The dopaminergic neuron proliferative progenitor cell marker polynucleotide probes of the present invention are used as markers for selecting and/or detecting dopaminergic neuron proliferative progenitor cells. Polynucleotides used for this probe comprise a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1 or 2 detected in dopaminergic neuron proliferative progenitor cells prior to cell division arrest. SEQ ID NO: 1 is the nucleotide sequence of murine Lrp4 cDNA. SEQ ID NO: 2 is the nucleotide sequence of human Lrp4 cDNA. Both sequences are registered in GenBank (murine: Accession No. NM_016869; human: Accession No. XM_035037).

[0020]

Here, a "marker polynucleotide probe" refers to a polymer composed of a number of nucleotides, such as deoxyribonucleic acids (DNAs) or ribonucleic acids (RNAs), or nucleotide pairs, where the polymer should be able to detect expression of Lrp4, particularly transcribed mRNA. Double-stranded cDNAs are also known to be able to be used as probes in tissue *in situ* hybridization, and such double-stranded cDNAs are included in the markers of the present invention. RNA probes (riboprobes) are particularly preferable as marker polynucleotide probes for detecting RNAs in tissue. If needed, the marker polynucleotide probes of the present invention can also contain non-naturally-occurring nucleotides such as 4-acetylcytidine, 5-(carboxyhydroxymethyl)uridine, 2'-O-methylcytidine, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluridine, dihydrouridine, 2'-O-methylpseudouridine, β-D-galactosylqueuosine, 2'-O-methylguanosine, inosine, N6-isopentenyladenosine, 1-methyladenosine, 1-methylpseudouridine, 1-methylguanosine, 1-methylinosine, 2,2-dimethylguanosine, 2-methyladenosine, 2-methylguanosine, 3-methylcytidine, 5-methylcytidine, N6-methyladenosine, 7-methylguanosine, 5-methylaminomethyluridine, 5-methoxyaminomethyl-2-thiouridine, β-D-mannosylqueuosine, 5-methoxycarbonylmethyl-2-thiouridine, 5-methoxycarbonylmethyluridine, 5-methoxyuridine, 2-methylthio-N6- isopentenyladenosine, N-((9-β-D-ribofuranosyl-2- methylthiopurin-6-yl)carbamoyl)threonine, N-((9-β-D-ribofuranosylpurin-6-yl)N-methylcarbamoyl)threonine, uridine-5-oxyacetic acid-methyl ester, uridine-5-oxyacetic acid, wybutoxosine, pseudouridine, queuosine, 2-thiocytidine, 5-methyl-2-thiouridine, 2-thiouridine, 4-thiouridine, 5-methyluridine, N-((9-β-D-ribofuranosylpurin-6-yl)carbamoyl)threonine, 2'-O-methyl-5-methyluridine, 2'-O-methyluridine, wybutosine, and 3-(3-amino-3-carboxy propyl)uridine.

[0021]

Moreover, marker polynucleotide probes of the present invention comprise nucleotide sequences complementary to nucleotide sequences encoding the amino acid sequence of SEQ ID NO: 3 or 4, encoding Lrp4 proteins. The nucleotide sequence that encodes the amino acid sequence of SEQ ID NO: 3 or 4 includes not only nucleotide sequences of SEQ ID NO: 1 or 2, but also nucleotide sequences that differ from the sequences of SEQ ID NO: 1 or 2 due to degeneracy of the genetic code. The marker polynucleotide probes of the present invention also include those which comprise sequences complementary to nucleotide sequences encoding a sequence that lacks the transmembrane domain in the amino acid sequence of SEQ ID NO: 3 or 4. There is no signal sequence in the amino acid sequence of SEQ ID NO: 3 or 4. In murine Lrp4 (SEQ ID NO: 3), amino acid residues 113-135 form a transmembrane domain, while in human Lrp4 (SEQ ID NO: 4), amino acid residues 46-68 form a transmembrane domain. The sequences described in SEQ ID NOs: 3 and 4 are respectively registered in GenBank (human Lrp4, XP_035037; murine Lrp4, NP_058565).

[0022]

Herein, the phrase "complementary to a nucleotide sequence" encompasses not only cases wherein a nucleotide sequence completely pairs with the template, but also includes those that have at least 70%, preferably 80%, more preferably 90%, and even more preferably 95% or more (for example, 97% or 99%) of the nucleotides paired with the template. Pairing refers to the formation of a chain in which T (U in the case of RNAs) corresponds to A, A corresponds to T or U, G corresponds to C, and C corresponds to G in the nucleotide sequence of the template polynucleotide. Homologies at the nucleotide sequence level between certain polynucleotides can be determined by the BLAST algorithm (Altschul (1990) Proc. Natl. Acad. Sci. USA 87: 2264-8; Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90: 5873-7). The BLASTN program for nucleotide sequences (Altschul *et al.* (1990) J. Mol. Biol. 215: 403-410) has been developed based on this algorithm, and can be used to determine the homology of marker polynucleotide probe sequences (see http://www.ncbi.nlm.nih.gov for a specific example of analysis methods).

[0023]

Moreover, marker polynucleotide probes of the present invention include polynucleotides that contain nucleotide sequences that hybridize under stringent conditions with polynucleotides comprised of the nucleotide sequence of SEQ ID NO: 1 or 2. Although polynucleotides with a nucleotide sequence indicated in SEQ ID NO: 1 or 2 are known with respect to Lrp4, alternative isoforms and allelic variants may also exist. Polynucleotides with a sequence complementary to such isoforms and allelic variants can also be used as marker polypeptides of the present invention. Such isoforms and allelic variants can be obtained

from cDNA libraries or genomic libraries derived from animals such as humans, mice, rats, rabbits, hamsters, chickens, pigs, cows, goats, and sheep, by using a polynucleotide probe comprising a nucleotide sequence of SEQ ID NO: 1 or SEQ ID NO: 2, in known hybridization methods such as colony hybridization, plaque hybridization, or Southern blotting. See "Molecular Cloning, A Laboratory Manual 2nd ed." (Cold Spring Harbor Press (1989)) for methods of cDNA library construction. In addition, commercially available cDNA libraries or genomic libraries may also be used.

[0024]

More specifically, in constructing a cDNA library, total RNA is first prepared from cells, organs, tissues, or such that express Lrp4, by known techniques such as guanidine ultracentrifugation (Chirwin *et al.* (1979) Biochemistry 18: 5294-5299) or AGPC (Chomczynski and Sacchi (1987) Anal. Biochem. 162: 156-159), followed by purification of mRNA using an mRNA Purification Kit (Pharmacia), or such. A kit for direct mRNA preparation, such as the QuickPrep mRNA Purification Kit (Pharmacia), may also be used. Next, cDNAs are synthesized from the resulting mRNAs using reverse transcriptase. cDNA synthesis kits, such as the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Seikagaku Corporation), are also commercially available. Other methods that use the 5'-RACE method to synthesize and amplify cDNA by PCR may also be used (Frohman *et al.* (1988) Proc. Natl. Acad. Sci. USA 85: 8998-9002; Belyavsky *et al.* (1989) Nucleic Acids Res. 17: 2919-32). In addition, in order to construct cDNA libraries containing a high percentage of full-length clones, known techniques such as the oligo-capping method (Maruyama and Sugano (1994) Gene 138: 171-4; Suzuki (1997) Gene 200: 149-56) can also be employed. The cDNA obtained in this manner is then incorporated into a suitable vector.

[0025]

Examples of hybridization conditions suitable for use in the present invention include "2x SSC, 0.1% SDS, 50°C", "2x SSC, 0.1% SDS, 42°C" and "1x SSC, 0.1% SDS, 37°C". Examples of conditions of higher stringency include "2x SSC, 0.1% SDS, 65°C", "0.5x SSC, 0.1% SDS, 42°C" and "0.2x SSC, 0.1% SDS, 65°C". More specifically, a method that uses the Rapid-hyb buffer (Amersham Life Science) can be carried out by performing pre-hybridization at 68°C for 30 minutes or more, adding a probe to allow hybrid formation at 68°C for one hour or more, washing three times in 2x SSC/0.1% SDS at room temperature for 20 minutes each, washing three times in 1x SSC/0.1% SDS at 37°C for 20 minutes each, and finally washing twice in 1x SSC/0.1% SDS at 50°C for 20 minutes each. This can also be carried out using, for example, the Expresshyb Hybridization Solution (CLONTECH), by performing pre-hybridization at 55°C for 30 minutes or more, adding a labeled probe and incubating at 37°C to 55°C for one hour or more, washing three times in 2x SSC/ 0.1% SDS at

room temperature for 20 minutes each, and washing once at 37°C for 20 minutes with 1x SSC/0.1% SDS. Herein, conditions of higher stringency can be achieved by increasing the temperature for pre-hybridization, hybridization, or the second wash. For example, pre-hybridization and hybridization temperatures of 60°C can be raised to 68°C for higher stringency. In addition to factors such as salt concentration of the buffer and temperature, those with ordinary skill in the art can also integrate other factors, such as probe concentration, probe length, and reaction time, to obtain Lrp4 isoforms and allelic variants, and corresponding genes derived from other organisms.

[0026]

References such as Molecular Cloning, A Laboratory Manual 2nd ed. (Cold Spring Harbor Press (1989); Section 9.47-9.58), Current Protocols in Molecular Biology (John Wiley & Sons (1987-1997); Section 6.3-6.4), DNA Cloning 1: Core Techniques, A Practical Approach 2nd ed. (Oxford University (1995); Section2.10 for conditions, in particular), can be referred to for detailed information on hybridization procedures. Examples of hybridizing polynucleotides include polynucleotides containing a nucleotide sequence that has at least 50% or more, preferably 70%, more preferably 80% and even more preferably 90% (for example, 95% or more, or 99%) identity with a nucleotide sequence comprising the nucleotides of SEQ ID NO: 1 or SEQ ID NO: 2. Such identities can be determined by the BLAST algorithm (Altschul (1990) Proc. Natl. Acad. Sci. USA 87: 2264-8; Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90: 5873-7) as described in the homology determination above. In addition to the above-described BLASTN program for nucleotide sequences, the BLASTX program for determining the identity of amino acid sequences (Altschul *et al.* (1990) J. Mol. Biol. 215: 403-10) and the like has been developed based on this algorithm and can be used (as described above, see http://www.ncbi.nlm.nih.gov. for a specific example of analysis methods).

[0027]

Lrp4 isoforms or allelic variants, and other genes with an Lrp4-like structure or function, can be obtained from cDNA libraries and genomic libraries of animals such as humans, mice, rats, rabbits, hamsters, chickens, pigs, cows, goats, and sheep, by designing primers based on the nucleotide sequences of SEQ ID NOs: 1 and 2, using gene amplification technology (PCR) (Current Protocols in Molecular Biology, John Wiley & Sons (1987) Sections 6.1-6.4).

[0028]

The polynucleotide sequences can be confirmed by using conventional sequence determination methods. For example, the dideoxynucleotide chain termination method (Sanger *et al.* (1977) Proc. Natl. Acad. Sci. USA 74: 5463) can be used. In addition,

sequences can also be analyzed using a suitable DNA sequencer.

[0029]

Moreover, marker polynucleotide probes of the present invention include the aforementioned (1) sequences complementary to the nucleotide sequence of SEQ ID NO: 1 or 2, (2) sequences complementary to nucleotide sequences that encode the amino acid sequence described in SEQ ID NO: 3 or 4, (3) sequences complementary to nucleotide sequences that encode a sequence lacking the transmembrane domain portion of the amino acid sequence described in SEQ ID NO: 3 or 4, and (4) polynucleotides comprising nucleotide sequences that contain at least 15 consecutive nucleotides in each of the nucleotide sequences that hybridize under stringent conditions with a polynucleotide comprised of the nucleotide sequence of SEQ ID NO: 1 or 2.

[0030]

Such polynucleotides comprising a nucleotide sequence that contains at least 15 consecutive nucleotides can be used as a probe for detecting, or as a primer for amplifying, the expression of Lrp4 mRNA. The nucleotide chain normally consists of 15 to 100, and is preferably 15 to 35 nucleotides when used as a probe, or at least 15 and preferably 30 nucleotides when used as a primer. A primer can be designed to have a restriction enzyme recognition sequence, a tag or such, added to the 5'-end side thereof, and at the 3' end, a sequence complementary to a target sequence. Such polynucleotides, comprising a nucleotide sequence that contains at lease 15 consecutive nucleotides, can hybridize with an Lrp4 polynucleotide.

[0031]

Marker polynucleotide probes of the present invention can be prepared from cells that express Lrp4 by the aforementioned hybridization or PCR or such. In addition, marker polynucleotide probes of the present invention can also be produced by chemical synthesis based on known Lrp4 sequence data. Riboprobes, which are considered to be particularly preferable for detecting RNA in tissues, can be obtained by, for example, inserting a cloned Lrp4 gene or portion thereof into plasmid vector pSP64 in the reverse direction, followed by run-off transcription of the inserted sequence portion. Although pSP64 contains an SP6 promoter, methods for producing riboprobes by combining phage T3, T7 promoter and RNA polymerase are also known.

[0032]

<Antibodies>

The present invention provides dopaminergic neuron progenitor cell marker antibodies which can be used to select dopaminergic neuron progenitor cells from brain tissue or cultured cells. Unlike Lrp4 mRNA, the Lrp4 polypeptide is expressed not only in the

dopaminergic neuron proliferative progenitor cells prior to cell division arrest, but also in postmitotic dopaminergic neuron progenitor cells. Therefore, by using the antibodies of the present invention against the polypeptide, it is possible to select/obtain dopaminergic neuron progenitor cells prior to and after cell division arrest. Antibodies of the present invention include polyclonal antibodies, monoclonal antibodies, chimeric antibodies, single-chain antibodies (scFV) (Huston *et al.* (1988) Proc. Natl. Acad. Sci. USA 85: 5879-83; The Pharmacology of Monoclonal Antibody, vol. 113, Rosenburg and Moore ed., Springer Verlag (1994) pp. 269-315), humanized antibodies, multispecific antibodies (LeDoussal *et al.* (1992) Int. J. Cancer Suppl. 7: 58-62; Paulus (1985) Behring Inst. Mitt. 78: 118-32; Millstein and Cuello (1983) Nature 305: 537-9; Zimmermann (1986) Rev. Physiol. Biochem. Pharmacol. 105: 176-260; Van Dijk *et al.* (1989) Int. J. Cancer 43: 944-9), and antibody fragments such as Fab, Fab', F(ab')2, and Fv. Moreover, antibodies of the present invention may also be modified by PEG and such, as necessary. Antibodies of the present invention may also be produced in the form of a fusion protein with β-galactosidase, maltose-binding protein, GST, green fluorescent protein (GFP) and such, to allow detection without the use of a secondary antibody. In addition, antibodies may be modified by labeling with biotin or such, to allow recovery using avidin, streptavidin, or such.

[0033]

The antibodies of present invention are specific to any of (1) polypeptides encoded by the nucleotide sequence of SEQ ID NO: 1 or 2, (2) polypeptides comprised of the amino acid sequence described in SEQ ID NO: 3 or 4, (3) polypeptides comprised of an amino acid sequence lacking the transmembrane domain in the amino acid sequence described in SEQ ID NO: 3 or 4, (4) polypeptides comprised of an amino acid sequence wherein one or more amino acids in the amino acid sequence of SEQ ID NO: 3 or 4 are deleted, inserted, substituted, or added, (5) polypeptides encoded by a nucleotide sequence that hybridizes under stringent conditions with a sequence complementary to the nucleotide sequence of SEQ ID NO: 1 or 2, and (6) polypeptides that are fragments of the polypeptides of (1) to (5) above, with at least eight amino acid residues.

[0034]

As the antibodies of the present invention, the two anti-Lrp4 antibodies used in Example 4, and modifications comprising fragments thereof, are particularly preferable. The two antibodies have been deposited under the following accession numbers:

(1) Name and address of the depositary institution

Name: International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology

Address: Central 6, 1-1-1 Higashi, Tsukuba-shi, Ibaraki Prefecture, Japan 305-8566

(2) Deposit date: July 14, 2004

(3) Accession Nos.: FERM AP-20120 and FERM AP-20121

[0035]

Antibodies of the present invention can be produced using a sensitizing antigen such as an Lrp4 polypeptide, or fragments thereof, or cells that express Lrp4 polypeptide or Lrp4 polypeptide fragments. In addition, short Lrp4 polypeptide fragments may also be used as immunogens by coupling with a carrier such as bovine serum albumin, Keyhole-limpet hemocyanin, and ovalbumin. In addition, the Lrp4 polypeptide, or fragments thereof, may be used in combination with known adjuvants, such as aluminum adjuvant, Freund's complete (or incomplete) adjuvant, or pertussis adjuvant, to enhance immune response to the antigen.

[0036]

The "Lrp4 polypeptide" in the present invention is a peptide polymer, a preferred example of which is a protein having the amino acid sequence described in SEQ ID NO: 3 or 4. The amino acid residues that compose an Lrp4 polypeptide may be naturally occurring or modified. Moreover, the Lrp4 polypeptides include proteins lacking a transmembrane domain portion, and fusion proteins modified by other peptide sequences.

[0037]

In the present invention, the Lrp4 polypeptides should have the antigenicity of the Lrp4 polypeptide, and include polypeptides with an amino acid sequence wherein one or more amino acids in the amino acid sequence of SEQ ID NO: 3 or 4 are deleted, inserted, substituted, or added. It is well known that mutant polypeptides comprising an amino acid sequence in which one or more amino acids are deleted, inserted, substituted, or added, maintain the same biological activity as the original polypeptide (Mark *et al.* (1984) Proc. Natl. Acad. Sci. USA 81: 5662-6; Zoller and Smith (1982) Nucleic Acids Res. 10: 6487-500; Wang *et al.* (1984) Science 224: 1431-3; Dalbadie-McFarland *et al.* (1982) Proc. Natl. Acad. Sci. USA 79: 6409-13). Such polypeptides that maintain the antigenicity of Lrp4 and have an amino acid sequence in which one or more amino acids are deleted, inserted, substituted, or added to the amino acid sequence of SEQ ID NO: 3 or 4, can be obtained by preparing polynucleotides that encode the polypeptides using known methods such as site-directed mutagenesis described in "Molecular Cloning, A Laboratory Manual 2nd ed." (Cold Spring Harbor Press (1989)), "Current Protocols in Molecular Biology" (John Wiley & Sons (1987-1997); especially section 8.1-8.5), Hashimoto-Goto *et al.* (1995) Gene 152: 271-5, Kunkel (1985) Proc. Natl. Acad. Sci. USA 82: 488-92, Kramer and Fritz (1987) Method. Enzymol. 154: 350-67, Kunkel (1988) Method. Enzymol. 85: 2763-6), and others, and then suitably expressing.

[0038]

Lrp4 polypeptide fragments are identical to a portion of the aforementioned Lrp4 polypeptide, and consists of at least eight amino acid residues or more (for example, 8, 10, 12, or 15 amino acid residues or more). A particularly preferred fragment can be exemplified by a polypeptide fragment lacking an amino terminus, carboxyl terminus, and transmembrane domain. The Lrp4 polypeptide fragments include fragments containing an α-helix and α-helix forming region, α-amphipathic region, β-sheet and β-sheet forming region, β-amphipathic region, substrate binding region, high antigen index region, coil and coil forming region, hydrophilic region, hydrophobic region, turn and turn forming region, and surface forming region. In the context of the present invention, an Lrp4 polypeptide fragment may be any fragment, so long as it has the antigenicity of an Lrp4 polypeptide. The antigen-determining site of a polypeptide can be predicted by using methods for analyzing the hydrophobicity/hydrophilicity of an amino acid sequence of a protein (Kyte-Doolittle (1982) J. Mol. Biol. 157: 105-22), or methods of secondary structure analysis (Chou-Fasman (1978) Ann. Rev. Biochem. 47: 251-76), and can be confirmed using computer programs (Anal. Biochem. 151: 540-6 (1985)), or the PEPSCAN method in which a short peptide is synthesized followed by confirmation of its antigenicity (JP-A (Kohyo) S60-500684), or the like.

[0039]

Lrp4 polypeptides and Lrp4 polypeptide fragments can be isolated from Lrp4-expressing cells, tissues, etc., based on their physical properties and such. In addition, these polypeptides and polypeptide fragments can also be produced using known genetic recombination techniques or chemical synthesis methods. For example, for *in vitro* Lrp4 polypeptide production, Lrp4 polypeptides can be produced in an *in vitro* cell-free system using methods such as *in vitro* translation (Dasso and Jackson (1989) Nucleic Acids Res. 17: 3129-44). In contrast, when producing polypeptides using cells, a polynucleotide that encodes a polypeptide of interest is first incorporated into an appropriate vector, a suitable host cell is selected, and then the cells are transformed by the vector. Subsequently, the transformed cells can be cultured to obtain the polypeptide of interest.

[0040]

Appropriate vectors include various vectors such as plasmids, cosmids, viruses, bacteriophages, cloning vectors, and expression vectors (Molecular Cloning, A Laboratory Manual 2nd ed., Cold Spring Harbor Press (1989); Current Protocols in Molecular Biology, John Wiley & Sons (1987)). The vectors comprise regulatory sequences for the expression of a desired polynucleotide in transfected host cells, and the polynucleotide is incorporated therein so that it will be under the control of the regulatory sequences. Here, the phrase "regulatory sequence" includes promoters, ribosome binding sites, and terminators in the case

of a prokaryotic host cell, and promoters and terminators in the case of a eukaryotic host cell, and in some cases, may also contain transactivators, transcription factors, poly A signals which stabilize transcription products, splicing and polyadenylation signals, and others. Such regulatory sequences comprise all the components required for the expression of a polynucleotide linked thereto. Vectors may further comprise a selection marker. Moreover, a signal peptide required for transferring an intracellularlly expressed polypeptide into the lumen of the endoplasmic reticulum, or the periplasm or extracellular space when the host is a Gram negative microbe, can also be incorporated into an expression vector by linking to a polypeptide of interest. Such signal peptides can be signal peptides derived from heterogeneous proteins. Moreover, a linker may be added, and a start (ATG) or stop codon (TAA, TAG, or TGA) may be inserted as necessary.

[0041]

Examples of vectors capable of expressing polypeptides *in vitro* include pBEST (Promega). In addition, various vectors are known to be suitable for expression in prokaryotic hosts (see, *e.g.*, "Basic Microbiology Course 8 - Genetic Engineering" (Kyoritsu Publishing)). When selecting prokaryotic cells as the host, a person with ordinary skill in the art can suitably select a vector suitable for the host and a method suitable for introducing the vector into the host. Other examples of hosts that can be used to express Lrp4 polypeptides and their antigenic fragments include fungal cells such as yeasts, higher plants, insects, fish, amphibians, reptiles, birds, mammals, cultured cells (COS, Hela, C127, 3T3, BHK, HEK293, Bowes melanoma cells), myeloma, Vero, Namalwa, Namalwa KJM-1, HBT5637 (JP-A (Kokai) S63-299), and such). Vector systems suitable for each cell and methods for introducing a vector into host cells are also known. Moreover, methods for expressing exogenous proteins in animals *in vivo* (see, *e.g.*, Susumu (1985) Nature 315: 592-4; Lubon (1998) Biotechnol. Annu. Rev. 4: 1-54) and in plant bodies are also known, and can be used to express Lrp4 polynucleotides.

[0042]

DNAs can be inserted into vectors in a ligase reaction using restriction enzyme sites (Current Protocols in Molecular Biology, John Wiley & Sons (1987) Section 11.4-11.11; Molecular Cloning, A Laboratory Manual 2nd ed., Cold Spring Harbor Press (1989) Section 5.61-5.63). In addition, an Lrp4 polypeptide-encoding expression vector can be designed as necessary by selecting a nucleotide sequence that has high expression efficiency in view of the host's codon usage frequency (Grantham *et al.* (1981) Nucleic Acids Res. 9: r43-74). A host that produces an Lrp4 polypeptide comprises in its cells a polynucleotide that encodes an Lrp4 polypeptide. So long as the polynucleotide does not exist at a naturally occurring position in the genome of a host cell, the polynucleotide itself may be regulated by its own promoter,

incorporated in the host genome, or maintained as an extrachromosomal structure.

[0043]

Culturing of host cells is carried out using known methods that are appropriate for the host cell selected. For example, when animal cells are selected, culturing can be carried out at a pH of about 6 to 8 and a temperature of 30°C to 40°C for about 15 to 200 hours, using a medium such as DMEM (Virology 8: 396 (1959)), MEM (Science 122: 501 (1952)), RPMI1640 (J. Am. Med. Assoc. 199: 519 (1967)), 199 (Proc. Soc. Biol. Med. 73: 1 (1950)), or IMDM, and adding serum such as fetal calf serum (FCS), as necessary. In addition, the medium may be replaced, aerated, or stirred during the course of culturing, as necessary.

[0044]

Normally, an Lrp4 polypeptide produced by gene recombination techniques is recovered from the medium if the polypeptide is secreted outside of a cell, or from the body fluid of a transgenic organism. When a polypeptide is produced inside of a cell, the cells are dissolved and the polypeptide is recovered from the dissolved product. The polypeptide of interest is then purified by suitably combining known methods of protein purification, such as salting out, distillation, various types of chromatography, gel electrophoresis, gel filtration, ultrafiltration, recrystallization, acid extraction, dialysis, immunoprecipitation, solvent precipitation, solvent extraction, and ammonium sulfate or ethanol precipitation. Examples of chromatographies include ion exchange chromatography, such as anion or cation exchange chromatography, affinity chromatography, reversed-phase chromatography, adsorption chromatography, gel filtration chromatography, hydrophobic chromatography, hydroxyapatite chromatography, phosphocellulose chromatography, and lectin chromatography (Strategies for Protein Purification and Characterization: A Laboratory Course Manual, Marshak *et al.* ed., Cold Spring Harbor Laboratory Press (1996)). Chromatography can be carried out using a liquid phase chromatography, such as HPLC or FPLC. In addition, for example, a protein fused with GST can be purified using a glutathione column, and a protein with a histidine tag can be purified using a nickel column. When an Lrp4 polypeptide is produced as a fusion protein, unnecessary portions can be removed using thrombin, factor Xa, or the like, following purification as necessary.

[0045]

In addition, naturally-occurring polypeptides can also be purified and obtained. For example, polypeptides can be purified by affinity chromatography using antibodies against the Lrp4 polypeptides (Current Protocols in Molecular Biology, John Wiley & Sons (1987) Section 16.1-16.19). Moreover, purified polypeptides can also be modified using enzymes, such as chymotrypsin, glucosidase, trypsin, protein kinase, and lysyl endopeptidase, as necessary. In addition to the aforementioned synthesis and genetic engineering techniques as

used for Lrp4 polypeptides, Lrp4 polypeptide fragments can also be produced by cleaving an Lrp4 polypeptide, using suitable enzymes, such as peptidases.

[0046]

Polyclonal antibodies for selecting dopaminergic neuron progenitor cells are obtained from, for example, the serum of an immunized animal after immunizing a mammal with an Lrp4 polypeptide purified as described above, or a fragment thereof, coupled to a desired adjuvant. Although there are no particular limitations on the mammals used, typical examples include rodents, lagomorphs, and primates. Specific examples include rodents such as mice, rats, and hamsters, lagomorphs such as rabbits, and primates such as monkeys, including cynomolgus monkeys, rhesus monkeys, baboons, and chimpanzees. Animal immunization is carried out by suitably diluting and suspending a sensitizing antigen in phosphate-buffered saline (PBS) or physiological saline, mixing with an adjuvant as necessary until emulsified, and injecting into an animal intraperitoneally or subcutaneously. The sensitizing antigen mixed with Freund's incomplete adjuvant is preferably administered several times, every 4 to 21 days. Antibody production can be confirmed by measuring the level of an antibody of interest in the serum using conventional methods. Finally, the serum itself may be used as a polyclonal antibody, or it may be further purified. See, for example, "Current Protocols in Molecular Biology" (John Wiley & Sons (1987) Sections 11.12-11.13), for specific methods.

[0047]

A monoclonal antibody is produced by removing the spleen from an animal immunized in the manner described above, separating immunocytes from the spleen, and fusing with a suitable myeloma cell using polyethylene glycol (PEG) or such to establish hybridomas. Cell fusion can be carried out according to the Milstein method (Galfre and Milstein (1981) Methods Enzymol. 73: 3-46). Here, suitable myeloma cells are exemplified particularly by cells that allow chemical selection of fused cells. When using such myeloma cells, fused hybridomas are selected by culturing in a culture medium (HAT culture medium) that contains hypoxanthine, aminopterin, and thymidine, which destroys cells other than fused cells. Next, clones that produce antibodies against polypeptides of the present invention, or a fragment thereof, is selected from the established hybridomas. Subsequently, the selected clone is introduced into the abdominal cavity of a mouse or such, and ascites is collected to obtain a monoclonal antibody. See also "Current Protocols in Molecular Biology" (John Wiley & Sons (1987) Section 11.4-11.11), for information on specific methods.

[0048]

Hybridomas can also be obtained by first sensitizing human lymphocytes that have been infected by EB virus with an immunogen *in vitro*, and fusing the sensitized lymphocytes

with human myeloma cells (such as U266) to obtain hybridomas that produce human antibodies (JP-A (Kokai) S63-17688). In addition, human antibodies can also be obtained by using antibody-producing cells generated by sensitizing a transgenic animal with a human antibody gene repertoire (WO92/03918; WO93/02227; WO94/02602; WO94/25585; WO96/33735; WO96/34096; Mendez *et al.* (1997) Nat. Genet. 15: 146-156, etc.). Methods that do not use hybridomas can be exemplified by a method in which a cancer gene is introduced to immortalize immunocytes such as antibody-producing lymphocytes.

[0049]

In addition, antibodies can also be produced by genetic recombination techniques (see Borrebaeck and Larrick (1990) Therapeutic Monoclonal Antibodies, MacMillan Publishers Ltd., UK). First, a gene that encodes an antibody is cloned from hybridomas or antibody-producing cells (such as sensitized lymphocytes). The resulting gene is then inserted into a suitable vector, the vector is introduced into a host, and the host is then cultured to produce the antibody. This type of recombinant antibody is also included in the antibodies of the present invention. Typical examples of recombinant antibodies include chimeric antibodies, comprising a non-human antibody-derived variable region and a human antibody-derived constant region, and humanized antibodies, comprising a non-human-derived antibody complementarity determining region (CDR), human antibody-derived framework region (FR), and human antibody constant region (Jones *et al.* (1986) Nature 321: 522-5; Reichmann *et al.* (1988) Nature 332: 323-9; Presta (1992) Curr. Op. Struct. Biol. 2: 593-6; Methods Enzymol. 203: 99-121 (1991)).

[0050]

Antibody fragments can be produced by treating the aforementioned polyclonal or monoclonal antibodies with enzymes such as papain or pepsin. Alternatively, antibody fragments can be produced by genetic engineering techniques using genes that encode antibody fragments (see Co *et al.*, (1994) J. Immunol. 152: 2968-76; Better and Horwitz (1989) Methods Enzymol. 178: 476-96; Pluckthun and Skerra (1989) Methods Enzymol. 178: 497-515; Lamoyi (1986) Methods Enzymol. 121: 652-63; Rousseaux *et al.* (1986) 121: 663-9; Bird and Walker (1991) Trends Biotechnol. 9: 132-7).

[0051]

Multispecific antibodies include bispecific antibodies (BsAb), diabodies (Db), and such. Multispecific antibodies can be produced by methods such as (1) chemically coupling antibodies having different specificities with different types of bifunctional linkers (Paulus (1985) Behring Inst. Mill. 78: 118-32), (2) fusing hybridomas that secrete different monoclonal antibodies (Millstein and Cuello (1983) Nature 305: 537-9), or (3) transfecting eukaryotic cell expression systems, such as mouse myeloma cells, with a light chain gene and

a heavy chain gene of different monoclonal antibodies (four types of DNA), followed by the isolation of a bispecific monovalent portion (Zimmermann (1986) Rev. Physio. Biochem. Pharmacol. 105: 176-260; Van Dijk *et al.* (1989) Int. J. Cancer 43: 944-9). Alternatively, diabodies are dimer antibody fragments comprising two bivalent polypeptide chains that can be constructed by gene fusion. These can be produced using known methods (see Holliger *et al.* (1993) Proc. Natl. Acad. Sci. USA 90: 6444-8; EP404097; WO93/11161).

[0052]

Recovery and purification of antibodies and antibody fragments can be carried out using Protein A and Protein G, or according to the protein purification techniques described above in producing non-antibody polypeptides (Antibodies: A Laboratory Manual, Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988)). For example, when using Protein A to purify antibodies of the present invention, Protein A columns such as Hyper D, POROS, or Sepharose F.F. (Pharmacia), are known and can be used. The concentration of the resulting antibodies can be determined by measuring absorbance or by enzyme linked immunosorbent assay (ELISA).

[0053]

The antigen binding activity of an antibody can be determined using absorbance measurements, or by using fluorescent antibody methods, enzyme immunoassay (EIA) methods, radioimmunoassay (RIA) methods, or ELISA. When ELISA is used, antibodies of the present invention are first immobilized onto a support, such as a plate. An Lrp4 polypeptide is added, and then a sample containing the antibody of interest is added. Herein, samples containing an antibody of interest include, for example, culture supernatants of antibody-producing cells, purified antibodies, and such. Next, secondary antibodies that recognize the antibodies of the present invention are added, and the plate is incubated. The plate is then washed and a label attached to the secondary antibody is detected. Namely, if a secondary antibody is labeled with alkaline phosphatase, antigen binding activity can be determined by adding an enzyme substrate such as p-nitrophenyl phosphate, and measuring absorbance. In addition, a commercially available system such as BIAcore (Pharmacia) can also be used to evaluate antibody activities.

[0054]

<Methods for Selecting Dopaminergic Neurons>

The present invention provides methods for selecting dopaminergic neuron proliferative progenitor cells prior to cell division arrest as a selectively uniform population. The dopaminergic neuron proliferative progenitor cells prior to cell division arrest can be particularly selected by using the marker polynucleotide probes of the present invention. The present invention also provides methods for selecting the dopaminergic neuron progenitor

cells including prior to and after cell division arrest as a selectively uniform population. The dopaminergic neuron progenitor cells including prior to and after cell division arrest can suitably be selected using the antibodies of the present invention. As described above, by using the polynucleotide probes or antibodies of the present invention, the dopaminergic neuron lineage cells which eventually differentiate into dopaminergic neurons can be specifically selected.

[0055]

Here, the term "selected" includes both detecting the presence of cells expressing markers in a sample, and subsequently separating or isolating those progenitor cells after detecting their presence. More specifically, the present invention provides methods for selecting dopaminergic neuron proliferative progenitor cells, comprising a step of contacting marker polynucleotide probes of the present invention with cell samples thought to be containing dopaminergic neuron proliferative progenitor cells. In this method, marker polynucleotide probes are preferably labeled with a radioactive isotope or non-radioactive compound. Examples of the radioactive isotopes used for labeling include ^{35}S and ^{3}H. When using a radiolabeled marker polynucleotide probe, RNA that binds with the marker can be detected by detecting silver particles using emulsion autoradiography. Examples of non-radioactive isotopes for labeling a marker polynucleotide probe include biotin and digoxigenin. A biotin-labeled marker can be detected using, for example, avidin labeled with fluorescence or an enzyme such as alkaline phosphatase or horseradish peroxidase. On the other hand, anti-digoxigenin antibodies labeled with fluorescence or an enzyme, such as alkaline phosphatase or horseradish peroxidase, can be used to detect a digoxigenin-labeled marker. When using enzyme labeling, detection is carried out by incubating with the enzyme substrate to form a stable pigment at the location of the marker. Fluorescent *in situ* hybridization (FISH) is convenient and particularly preferable.

[0056]

In addition, the present invention provides methods for selecting dopaminergic neurons comprising the step of contacting antibodies for selecting the dopaminergic neuron progenitor cells of the present invention with cell samples thought to be containing dopaminergic neuron progenitor cells. More specifically, cells expressing Lrp4 polypeptides, namely dopaminergic neuron progenitor cells containing prior to and after cell division arrest, can be acquired by contacting cell samples containing potential dopaminergic neuron progenitor cells with antibodies of the present invention, and selecting those cells that have bound to the antibody (see Fig. 13). The antibodies may also be immobilized on a suitable support, prior to contact with cells. Alternatively, cells that bind with the antibodies can be selectively recovered by contacting cells with the antibodies, allowing them to bind, and then

purifying the antibody by affinity chromatography. For example, if the antibodies of the present invention are conjugated to biotin, they can be purified on a plate or column bound with avidin or streptavidin. In addition, magnetic particles can be bound to an antibody, for example, and the antibody and cells that express Lrp4 on their surfaces, where the Lrp4 is bound to the antibody, can be recovered using a magnet. Dopaminergic neurons that express Lrp4 can be selected by flow cytometry using a cell sorter and fluorescent-labeled anti-Lrp4 antibodies and such.

[0057]

Furthermore, according to the present invention, the dopaminergic neuron progenitor cells, which have a low risk of tumorigenesis and are suitable for transplant therapy, can be obtained by culturing or not culturing the dopaminergic neuron progenitor cells selected by using the marker polynucleotide probes or antibodies of the present invention; and further screening by using the postmitotic dopaminergic neuron markers. The postmitotic dopaminergic neuron progenitor cell markers can include, for example, 65B13, MAP2, Nurr1, and TH (WO 2004/038018; Kawasaki *et al.*, (2000) Neuron 28: 31-40; Wallen *et al.*, (1999) Exp. Cell Res., 253: 737-46). For example, the postmitotic dopaminergic neuron progenitor cells can be selected by contacting the antibody against 65B13 polypeptide with the dopaminergic neuron progenitor cells selected by using the marker polynucleotide probes or antibodies of the present invention or further cultured as necessary to select the cells expressing the 65B13 polypeptides. Furthermore, 65B13 has an Ig-domain adhesion molecule-like structure. When 65B13 is expressed in cultured cells, cells expressing 65B13 adhere together, but do not adhere to cells without expressing 65B13. Thus, it is expected that adhesion *via* 65B13 is a homophilic binding. Then, dopaminergic neuron progenitor cells expressing 65B13 can be screened by utilizing binding of extracellular domain of 65B13 polypeptide.

[0058]

In addition, Lrp4-expressing dopaminergic neuron proliferative progenitor cells and 65B13-expressing dopaminergic neuron progenitor cells can also be selected and/or screened using promoters for Lrp4 and 65B13, respectively (see, for example, JP-A (Kokai) 2002-51775). For example, a vector harboring a construct that comprises a gene encoding a detection marker, such as GFP, linked to a promoter region obtained from analyzing the Lrp4 expression regions to be described later, can be transfected into cells. In addition, a gene encoding a marker can also be knocked in at the Lrp4 gene locus. In either case, specific cells can be selected by detecting the expression of a marker gene specific to dopaminergic neuron progenitor cells. As for 65B13, screening can also be conducted by the same methods as for Lrp4. For 65B13, for example, sequences described in WO 2004/038018 can

be referred to.

[0059]

The cell samples used herein preferably comprise cells of the ventral midbrain region or culture medium containing *in vitro* differentiated dopaminergic neurons. *In vitro* differentiation of dopaminergic neurons can be carried out by known methods using a starting material like cells such as known ES cells, bone marrow interstitial cells, immortalized neuron-derived cell lines (JP-A (Kohyo) H08-509215; JP-A (Kohyo) H11-506930; JP-A (Kohyo) 2002-522070), or primordial neuron cells (JP-A (Kohyo) H11-509729). Normally, dopaminergic neurons can be differentiated by co-culturing a tissue obtained from a dopaminergic neuron region of the brain, with a sustentacular cell layer derived from neural tissues. Moreover, methods are also known for deriving dopaminergic cells from neural tissues that do not normally produce dopamine, such as the striatum and cortex (JP-A (Kohyo) H10-509319). In addition, culturing under hypoxic conditions has been reported to produce cells containing a greater number of dopaminergic neurons (JP-A (Kohyo) 2002-530068). Cell samples used in the selection of dopaminergic neuron progenitor cells of the present invention may be cell populations isolated or cultured by any method, including the above-described methods.

[0060]

In addition, the supports used in immobilizing the antibodies or polypeptides of the present invention are necessarily safe for cells. Examples of such supports include synthetic or naturally-occurring organic polymer compounds, inorganic materials such as glass beads, silica gel, alumina, and activated charcoal, and those with surfaces coated with a polysaccharide or synthetic polymer. There are no particular limitations on the form of the support, examples of which include films, fibers, granules, hollow fibers, non-woven fabrics, porous supports, or honeycombed supports. The contact surface area of the supports can be controlled by changing their thickness, surface area, width, length, shape, and size in various ways.

[0061]

<Dopaminergic Neuron Progenitor Cells>

The cells acquired using polynucleotide probes and expression of Lrp4 mRNA as an index are dopaminergic neuron proliferative progenitor cells prior to cell division arrest. Cells acquired using the antibodies and expression of Lrp4 polypeptides as an index are dopaminergic neuron progenitor cells prior to and after cell division arrest. Thus, by using either the mRNAs or the polypeptides as indexes, cell populations with dopamine neuron lineage cell alone can be obtained. The progenitor cells obtained by the methods of the present invention are more preferable for transplant therapy for diseases related to postural

reflex, movement, and reward-associated behaviors, particularly neurodegenerative diseases such as PD, schizophrenia, and drug habits (Hynes *et al.*, (1995) Cell 80: 95-101) in terms of safety, survival rate, and network forming capacity compared to conventional unpurified cell populations or dopaminergic neurons into which exogenous genes are introduced. The cells acquired using Lrp4 expression as an index can be used for transplantation directly or after *in vitro* proliferation (Fig. 13). Such cells are expected to exert therapeutic effects because they are likely to differentiate and mature in an optimal place in the brain. In particular, since the dopaminergic neuron progenitor cells of the present invention selected using Lrp4 mRNA expression as an index are proliferating progenitor cells, and are likely to further proliferate *in vivo*, they are expected to exert therapeutic effects for an extended period. Furthermore, the cells (cell populations) of the present invention obtained by the methods of the present invention using the Lrp4 as an index can be differentiated *in vitro* to an appropriate stage by selecting conditions such as the medium, and are preferred as the materials for various neural transplant therapies. As described above, for example, the cells selected using Lrp4 expression as an index can be subjected to further selection using markers (e.g., 65B13, MAP2, Nurr1, TH, etc.) for postmitotic dopaminergic neurons, to obtain even safer cells for use in transplantation.

[0062]

1×10^3 to 1×10^6 dopaminergic neuron progenitor cells obtained using the methods of the present invention can be transplanted, more preferably 5×10^4 to 6×10^4 cells. The primary method is stereotaxic surgery, in which a cell suspension is transplanted into the brain. In addition, cells may also be transplanted by microsurgery. For methods of transplanting neuron tissues see Backlund *et al.* (Backlund *et al.* (1985) J. Neurosurg. 62: 169-73), Lindvall *et al.* (Lindvall *et al.* (1987) Ann. Neurol. 22: 457-68), or Madrazo *et al.* (Madrazo *et al.* (1987) New Engl. J. Med. 316: 831-4).

[0063]

Moreover, the cells of the present invention can also be used to isolate genes specific to dopaminergic neuron progenitor cells, and genes specific to each stage of maturation from progenitor cells into dopaminergic neurons. They can also be used to search for therapeutic targets for PD, to elucidate the maturation process of dopaminergic neurons, in screenings using maturation as an indicator, and such.

[0064]

<Comparison of Gene Expression Levels>

Dopaminergic neuron progenitor cells, obtained using the polynucleotide probes and antibodies of the present invention, can be used as materials to isolate genes specifically expressed in these cells. They can also be used to investigate and isolate genes specifically

expressed in cells that have been differentiated, induced, or proliferated from the dopaminergic neuron progenitor cells of the present invention. In addition, they can also be used to investigate the genes required for *in vivo* differentiation of dopaminergic neurons, by investigating genes that have different expression levels in cells that have differentiated, induced, or proliferated and the original progenitor cells. Since such genes are potential candidates for treating diseases caused by defects in dopaminergic neurons, determining and isolating them is extremely useful.

[0065]

Comparison of gene expression levels in the dopaminergic neuron progenitor cells of the present invention with those in cells that have been differentiated, induced, or proliferated therefrom, or other cells; or comparison of gene expression levels of the differentiated, induced, or proliferated cells with those of other cells, can be done using commonly used methods, such as cell *in situ* hybridization, Northern blot hybridization, RNA dot blot hybridization, reverse transcription PCR, RNase protection assay, DNA microarray hybridization, serial analysis of gene expression (SAGE) (Velculescu *et al.* (1995) Science 270: 484-487), subtractive hybridization, and representation difference analysis (RDA) (Lisitsyn (1995) Trends Genet. 11: 303-307).

[0066]

For cellular *in situ* hybridization, places where RNA processing, transport, and localization into the cytoplasm occur in individual cells can be investigated, by hybridizing total RNA or poly A^+ RNA prepared from cells with a labeling probe specific to a given RNA sequence. In addition, RNA size can be determined from size fractioning using gel electrophoresis. Moreover, RNA transcription products can be visualized *in situ* by using quantitative fluorescent *in situ* hybridization (FISH) and a digital imaging microscope (Femino *et al.* (1998) Science 280: 585-90), which are applicable to the present invention.

[0067]

When using reverse transcription PCR for gene expression analysis, the expression of specific genes can be roughly quantified. Various isoforms of a single RNA transcription product can also be detected and analyzed using the present methods. For reverse transcription PCR, when the reaction is carried out using exon-specific primers, and amplification products other than the predicted product are detected, mRNA isoforms produced by alternative splicing can be identified by analyzing these products. For more details see, for example, the method described in Pykett *et al.* (1994) Hum. Mol. Genet. 3: 559-64. When a quick and rough analysis of expression pattern is required, the methods which use PCR of the present invention are particularly preferred, in terms of their high speed, high sensitivity, and simplicity.

[0068]

The efficiency of gene expression screening can be improved by using DNA chips. Herein, a DNA chip refers to a miniature array in which oligonucleotides, DNA clones, or such are immobilized at a high density on a support surface, such as glass. For example, in order to carry out multiple expression screening, cDNA clones for each gene of interest, or oligonucleotides specific to each gene, are immobilized on a chip to produce a microarray. Next, RNAs are prepared from the dopaminergic neuron progenitor cells of the present invention, or cells differentiated, induced, or proliferated therefrom, and treated with reverse transcriptase to yield cDNAs. Next, the resulting cDNA samples are labeled with fluorescent tags or other tags, and then hybridized to the microarray. As a result, genes that are actively expressed in the cells have a higher percentage of total labeled cDNA, while genes that are not significantly expressed have a lower percentage. Namely, the fluorescent signal intensity, which represents hybridization between a labeled cDNA and a cDNA clone or an oligonucleotide on the chip, reflects the expression level of each sequence in the labeled cDNA, and thereby enables quantification of gene expression.

[0069]

In addition, multiple genes in the dopaminergic neuron progenitor cells of the present invention, or cells differentiated, induced, or proliferated therefrom, can be simultaneously analyzed by mRNA differential display, which involves reverse transcription PCR using degenerate PCR primers. First, a modified oligo dT primer is prepared, in which one or two nucleotides at the 3' terminus in the poly A tail of a given mRNA have been altered. Then, a reverse transcription reaction is carried out using the total RNAs isolated from the progenitor cells of the present invention, cells differentiated or proliferated therefrom, or control cells to be used for expression comparison (Liang *et al.* (1993) Nucleic Acids Res. 21: 3269-3275). If the altered nucleotide is a "G", then mRNAs with a "C" immediately before the poly A tail can be selectively amplified. If the altered nucleotides are "CA", then mRNAs with "TG" immediately before the poly A tail can be selectively amplified. Next, an arbitrary nucleotide sequence of about 10 nucleotides in length is prepared for use as a second primer, and a PCR amplification reaction is carried out using the modified oligo dT primer and this second primer. The amplification product is subjected to size fractionation by electrophoresis using a long polyacrylamide gel. By using such methods, cDNAs derived from the mRNAs specifically expressed in either of the cells of the present invention or the control cells can be detected as bands only present in the samples that have been electrophoresed. These methods can also be used to analyze expression of unidentified genes.

[0070]

SAGE analysis does not require a special device for detection, and is one of the

preferred analytical methods for simultaneously detecting the expression of a large number of transcription products. First, poly A^+ RNA is extracted from the dopaminergic neuron progenitor cells of the present invention, or cells differentiated, induced, or proliferated therefrom, using standard methods. Next, the RNAs are converted into cDNAs using a biotinylated oligo (dT) primer, and are then treated with a four-base recognizing restriction enzyme (Anchoring Enzyme: AE). Here, the AE-treated fragments contain a biotin group at their 3' terminus. Next, the AE-treated fragments are incubated with streptavidin for binding. The bound cDNA is divided into two fractions, and each fraction is then linked to a different double-stranded oligonucleotide adapter (linker) A or B. These linkers are composed of: (1) a protruding single strand portion having a sequence complementary to the sequence of the protruding portion formed by the action of the anchoring enzyme, (2) a 5' nucleotide recognizing sequence of the IIS-type restriction enzyme (cleaves at a predetermined location no more than 20 bp away from the recognition site) serving as a tagging enzyme (TE), and (3) an additional sequence of sufficient length for constructing a PCR-specific primer. Herein, the linker-linked cDNA is cleaved using the tagging enzyme, and only the linker-linked cDNA sequence portion remains, which is present in the form of a short-strand sequence tag. Next, pools of short-strand sequence tags from the two different types of linkers are linked to each other, followed by PCR amplification using primers specific to linkers A and B. As a result, the amplification product is obtained as a mixture comprising myriad sequences of two adjacent sequence tags (ditags) bound to linkers A and B. The amplification product is treated with the anchoring enzyme, and the free ditag portions are linked into strands in a standard linkage reaction. The amplification product is then cloned. The clone's determined nucleotide sequence can be used to obtain a read-out of consecutive ditags of constant length. The presence of mRNA corresponding to each tag can then be identified once from the determination of the clone's nucleotide sequence and information on the sequence tags thus obtained.

[0071]

Subtraction hybridization is frequently used to clone genes with different expression levels in various tissues or cells, and can also be used to clone genes specifically expressed in the dopaminergic neuron progenitor cells of the present invention, or cells differentiated, induced, or proliferated therefrom. First, from the progenitor cells of the present invention, a DNA sample of a cell to be tested is prepared (hereinafter referred to as "test DNA"). Next, a DNA of a cell to be compared is prepared (hereinafter referred to as "driver DNA"). The test and driver DNAs can also be used interchangeably. In any case, genes present in the test DNA but absent from the driver DNA are detected. Next, the prepared test DNA is mixed with a large excess of driver DNA, denatured to form single-stranded DNA, then annealed.

By regulating the annealing conditions, specific sequences absent from the driver DNA can be isolated as double-stranded DNAs comprising only the test DNA sequence. For further detail on this method see, Swaroop *et al.* (1991) Nucleic Acids Res. 19: 1954 and Yasunaga *et al.* (1999) Nature Genet. 21: 363-9.

[0072]

The RDA method is a method that uses PCR to selectively amplify a sequence of a test DNA that is absent in a driver DNA, and can be used in the present invention similarly to other previously described methods. For more details on the procedure see Lisitsyn (1995) Trends Genet. 11: 303-7 and Schutte *et al.* (1995) Proc. Natl. Acad. Sci. USA 92: 5950-4.

[0073]

Genes specific to dopaminergic neuron progenitor cells, or cells differentiated, induced, or proliferated therefrom, are detected and isolated as described, and can be inserted into vectors or such, for sequence determination and expression analysis using the various known methods described above.

[0074]

<Screening Using Progenitor Cell Maturation as an Index>

The present invention provides screening methods that comprise the step of contacting test substances with the dopaminergic neuron progenitor cells of the present invention, and the step of detecting the differentiation or proliferation of the progenitor cells that results from that contact. Since compounds obtained by this screening method demonstrate a regulatory function in the differentiation, proliferation, and such, of dopaminergic neurons, they are considered useful as potential therapeutic candidates for diseases caused by defects in dopaminergic neurons. The dopaminergic neuron progenitor cells of the present invention include cells selected by using the polynucleotide probes or antibodies of the present invention, and cells obtained by the proliferation/differentiation induction of these cells.

[0075]

Here, the "test substance" may be any type of compound, examples of which include the expression products of gene libraries, synthetic low molecular weight compound libraries, synthetic peptide libraries, antibodies, substances released by bacteria, cell (microbial, plant, or animal) extracts, cell (microbial, plant, or animal) culture supernatants, purified or partially purified polypeptides, marine organisms, plant or animal extracts, soil, random phage peptide display libraries, and such.

[0076]

Cell differentiation and proliferation can be detected by comparison with cell status in the absence of the test substance. Cell differentiation and proliferation may be detected by

morphological observation under a microscope or by detection or quantification of substances produced in cells, such as dopamine.

[0077]

<Analysis of Lrp4 Expression Region>

Using a sequence of the Lrp4 gene, an expression regulatory region of Lrp4 can be cloned from genomic DNA by known methods. For example, a method for establishing the transcriptional start site, such as the S1 mapping method, is known and can be used (Cell Engineering, Supplement 8, New Cell Engineering Experiment Protocol, Cancer Research Division, The Institute of Medical Science, The University of Tokyo ed., Shujunsha Publishing (1993) pp. 362-374). In general, the expression regulatory region of a gene can be cloned by screening genomic DNA libraries, using probe DNAs comprising a 15-100 bp segment, and preferably a 30-50 bp segment, of the gene's 5' terminus (in the present invention, all or a portion of nucleotides of SEQ ID NO: 1 or SEQ ID NO: 2). A clone obtained in this manner contains a 5' non-coding region of 10 kbp or more, and is shortened or fragmented by exonuclease treatment, or such. Finally, the shortened sequence portion, comprising a potential expression regulatory region, is evaluated for the strength, regulation and such of its expression using a reporter gene, thereby making it possible to determine the minimum unit required to maintain the activity of the Lrp4 expression regulatory region.

[0078]

Gene expression regulatory regions can be predicted using a program such as Neural Network (http://www.fruitfly.org./seq_tools/promoter.html; Reese *et al.*, Biocomputing: Proceedings of the 1996 Pacific Symposium, Hunter and Klein ed., World Scientific Publishing Co., Singapore, (1996)). Moreover, a program for predicting the minimum unit required for the activity of an expression regulatory region is also known and can be used (http://biosci.cbs.umn.edu./software/proscan/promoterscan. htm; Prestridge (1995) J. Mol. Biol. 249: 923-932).

[0079]

The expression region of the Lrp4 gene isolated in this manner can be used to produce proteins of interest specifically in dopaminergic neuron proliferative progenitor cells prior to cell division arrest *in vivo*.

[0080]

<Ligand for Lrp4>

The Lrp4 polypeptides have a transmembrane domain, and thus in nature are thought to exist embedded within the cell membrane. Due to its expression in dopaminergic neuron progenitor cells prior to and after cell division arrest, Lrp4 is believed to be involved in the regulation of progenitor cell proliferation and in neuron differentiation and maturation. Thus,

potential ligands that may demonstrate an agonistic or antagonistic function towards Lrp4 may be used to regulate the differentiation of dopaminergic neurons *in vivo*, *ex vivo*, and *in vitro*. In identifying ligands for Lrp4 polypeptides, an Lrp4 polypeptide and a candidate compound are first contacted and tested for the presence of binding. In this case, the Lrp4 polypeptide can be used when immobilized on a support, or embedded in the cell membrane. There are no particular limitations on the candidate compounds, examples of which include expression products of gene libraries, natural substances derived from marine organisms, extracts of various types of cells, known compounds and peptides, natural substances derived from plants, body tissue extracts, microbial culture supernatants and peptide groups randomly produced by the phage display method (J. Mol. Biol. 222: 301-10 (1991)). In addition, the candidate compound may be labeled for detection of binding.

[0081]

<Inhibition of Lrp4 Expression>

Since the present invention clearly demonstrates that Lrp4 mRNA is transiently expressed in dopaminergic neuron proliferative progenitor cells prior to cell division arrest, Lrp4 may be involved in the control of progenitor cell proliferation as well as neuron differentiation and maturation. Thus, substances that inhibit Lrp4 gene expression may be used to control the differentiation of dopaminergic neurons *in vivo*, *ex vivo*, and *in vitro*. Examples of substances capable of inhibiting gene expression include antisense nucleic acids, ribozymes, and double-stranded RNAs (small interfering RNA; siRNA). Thus, the present invention provides such antisense nucleic acids, ribozymes, and double-stranded RNAs.

[0082]

Examples of antisense mechanisms that suppress target gene expression include: (1) inhibition of transcription initiation via triplex formation, (2) transcription suppression through hybrid formation at sites of local open-loop structures formed by RNA polymerase, (3) transcription inhibition through hybrid formation with RNA during synthesis, (4) suppression of splicing through hybrid formation at intron-exon junctions, (5) suppression of splicing through hybrid formation at sites of spliceosome formation, (6) suppression of mRNA migration to the cytoplasm through hybrid formation with mRNA, (7) suppression of splicing through hybrid formation at a capping site or poly A addition site, (8) suppression of translation initiation through hybrid formation at binding sites of translation initiation factors, (9) translation suppression through hybrid formation at ribosome binding sites, (10) suppression of peptide chain elongation through hybrid formation at mRNA coding regions or polysome binding sites, and (11) suppression of gene expression through hybrid formation at sites of nucleic acid/protein interaction (Hirashima and Inoue, "New Biochemistry Experiment Course 2, Nucleic Acids IV, Gene Replication and Expression", Japanese Biochemical Society

edit., Tokyo Kagaku Dozin Publishing, pp. 319-347 (1993)).

[0083]

An Lrp4 antisense nucleic acid of the present invention may be a nucleic acid that inhibits gene expression by any of the mechanisms described in (1) to (11) above. Namely, it may contain an antisense sequence to not only a sequence of a coding region, but also a sequence of a non-coding region of a target gene whose expression is to be inhibited. A DNA that encodes an antisense nucleic acid can be used by linking to a suitable regulatory sequence that allows its expression. The antisense nucleic acid does not need to be completely complementary to the coding region or non-coding region of a target gene, as long as it can effectively inhibit the expression of this gene. Such antisense nucleic acids have a chain length of at least 15 bp or more, preferably 100 bp or more, and more preferably 500 bp or more, and are normally within 3,000 bp, preferably within 2,000 bp, and more preferably within 1,000 bp. It is preferable that such antisense nucleic acids share an identity of 90% or more, and more preferably 95% or more, with the complementary chain of a target gene transcription product. These antisense nucleic acids can be prepared according to phosphorothionate methods (Stein (1988) Nucleic Acids Res. 16: 3209-21) or the like, using Lrp4 polynucleotides.

[0084]

"Ribozyme" is a generic term referring to catalysts with an RNA component, and ribozymes are broadly classified into large ribozymes and small ribozymes. Large ribozymes cleave the phosphate-ester bonds of a nucleic acid, and after reaction, they leave 5'-phosphoric acid and 3'-hydroxyl group at the reaction sites. Large ribozymes are further classified into (1) group I intron RNAs, which carry out guanosine-initiated trans-esterification reactions at 5'-splice sites, (2) group II intron RNAs, which perform two-step self-splicing reactions via a lariat structure, and (3) RNA components of ribonuclease P, which cleave precursor tRNAs at their 5' side *via* hydrolysis reactions. In contrast, small ribozymes are comparatively small structural units (about 40 bp) that cleave RNAs, forming 5'-hydroxyl groups and 2'-3' cyclic phosphoric acids. Small ribozymes include, for example, hammerhead-type ribozymes (Koizumi *et al.* (1988) FEBS Lett. 228: 225) and hairpin-type ribozymes (Buzayan (1986) Nature 323: 349; Kikuchi and Sasaki (1992) Nucleic Acids Res. 19: 6751; Kikuchi (1992) Chemistry and Biology 30: 112). Since ribozymes are easily altered and synthesized, various methods for their modification are known. For example, hammerhead-type ribozymes that recognize and cleave nucleotide sequence UC, UU, or UA within a target RNA can be created, by designing the substrate binding portion of a ribozyme to be complementary to an RNA sequence near the target site (Koizumi *et al.* (1988) FEBS Lett. 228: 225; M. Koizumi and E. Ohtsuka (1990) Protein, Nucleic Acid and Enzyme 35: 2191; Koizumi *et al.* (1989) Nucleic

Acids Res. 17: 7059). Hairpin-type ribozymes can also be designed and produced using known methods (Kikuchi and Sasaki (1992) Nucleic Acids Res. 19: 6751; Kikuchi (1992) Chemistry and Biology 30: 112).

[0085]

Antisense nucleic acids and ribozymes of the present invention can also be used in viral vectors derived from retroviruses, adenoviruses, adeno-associated viruses, and such, or non-viral vectors that use liposomes, or naked DNAs, to control gene expression in cells using *ex vivo* or *in vivo* gene therapy.

[0086]

In 1998, a phenomenon that RNAs interfere each other and lose their function (RNA interference) was observed in *C. elegans* (Fire *et al.* (1998) Nature 391: 806-11). RNA interference is a phenomenon in which the introduction of an artificial double-stranded RNA into cells causes RNAs having the same nucleotide sequence to be degraded. The later study suggests that RNA silencing phenomenon such as RNA interference is cellular mechanisms for excluding defective mRNAs, and protecting from transposons or parasites such as viruses. Recently, double-stranded RNAs (small interfering RNAs; siRNAs) are utilized as tools for suppressing expressions of many genes, and methods for treating/preventing diseases are studied by suppressing expressions of genes responsible for the diseases using siRNAs. There are no particular limitations on the siRNAs of the present invention, provided they inhibit transcription of Lrp4 mRNA. Normally, an siRNA is a combination of a sense and antisense chain to the sequence of a target mRNA, and has a nucleotide length of at least 10 to the same number of nucleotides as the target mRNA. These siRNAs preferably have a nucleotide length of 15 to 75, preferably 18 to 50, and more preferably 20 to 25 nucleotides.

[0087]

In order to suppress Lrp4 expression, siRNAs can be introduced into cells using known methods. For example, a DNA is designed to encode, in a single strand, two RNA chains that compose an siRNA, and this is then incorporated into an expression vector, cells are transformed with the expression vector, and the siRNA can be expressed in the cells in the form of a double-stranded RNA with a hairpin structure. Plasmid expression vectors that continuously produce siRNA by transfection have also been designed (for example, RNAi-Ready pSIREN Vector, and RNAi-Ready pSIREN-RetroQ Vector (BD Biosciences Clontech)).

[0088]

The nucleotide sequence of an siRNA can be designed using a computer program such as that disclosed at the Ambion website (http://www.ambion.com/techlib/misc/siRNA_finder.html). Kits for screening for functional siRNAs are also commercially available and can be used (for example, BD Knockout RNAi

System (BD Biosciences Clontech).

[Examples]

[0089]

The present invention will be specifically described using Examples, but it is not be construed as being limited thereto.

[0090]

[Example 1] Isolation and Sequence Analysis of a Gene Specific to Dopaminergic Neuron Progenitor Cells

To isolate genes specific to dopaminergic neuron progenitor cells, the midbrain ventral region of E12.5 mice was additionally cut into two regions in the dorsoventral direction, and genes specifically expressed in the most ventral region containing dopaminergic neurons were identified by the subtraction (N-RDA) method. One of the isolated cDNA fragments was a fragment encoding Lrp4/Corin. Lrp4 encodes type II transmembrane proteins (Fig. 1).

[0091]

(1) N-RDA Method

(1)-1. Adapter Preparation

The following oligonucleotides were annealed to each other, and prepared at 100 μM. (ad2: ad2S+ad2A, ad3: ad3S+ad3A, ad4: ad4S+ad4A, ad5: ad5S+ad5A, ad13: ad13S+ad13A)

ad2S: cagctccacaacctacatcattccgt (SEQ ID NO: 5)

ad2A: acggaatgatgt (SEQ ID NO: 6)

ad3S: gtccatcttctctctgagactctggt (SEQ ID NO: 7)

ad3A: accagagtctca (SEQ ID NO: 8)

ad4S: ctgatgggtgtcttctgtgagtgtgt (SEQ ID NO: 9)

ad4A: acacactcacag (SEQ ID NO: 10)

ad5S: ccagcatcgagaatcagtgtgacagt (SEQ ID NO: 11)

ad5A: actgtcacactg (SEQ ID NO: 12)

ad13S: gtcgatgaacttcgactgtcgatcgt (SEQ ID NO: 13)

ad13A: acgatcgacagt (SEQ ID NO: 14)

[0092]

(1)-2. cDNA Synthesis

Ventral midbrain regions were cut out of E12.5 mouse embryos (Japan SLC), and divided into two sections in the dorsoventral direction. Total RNA was prepared using the RNeasy Mini Kit (Qiagen), and double-stranded cDNA was synthesized using a cDNA Synthesis Kit (Takara). After digestion with restriction enzyme RsaI, ad2 was added. The cDNA was amplified by a 5-minute incubation at 72°C, 15 PCR cycles of 30 seconds at 94°C,

30 seconds at 65°C, and 2 minutes at 72°C, and a final 2-minute incubation at 72°C using ad2S as the primer. In all cases, N-RDA PCR was carried out using a reaction solution containing the following components.

10x ExTaq	5 μl
2.5 mM dNTP	4 μl
ExTaq	0.25 μl
100 μM primer	0.5 μl
cDNA	2 μl
Distilled water	38.25 μl

[0093]

(1)-3. Driver Preparation

The ad2S-amplified cDNA was further amplified by incubating at 94°C for 2 minutes, and then performing five PCR cycles of 30 seconds at 94°C, 30 seconds at 65°C, and 2 minutes at 72°C, and a final 2-minute incubation at 72°C. The cDNA was purified using the Qiaquick PCR Purification Kit (Qiagen), and digested with RsaI. 3 μg was used for each round of subtraction.

[0094]

(1)-4. Tester Preparation

The ad2S amplified cDNA was further amplified by incubating at 94°C for 2 minutes, and then performing five PCR cycles of 30 seconds at 94°C, 30 seconds at 65°C, and 2 minutes at 72°C, and a final 2-minute incubation at 72°C. The cDNA was purified using the Qiaquick PCR Purification Kit (Qiagen), and digested with RsaI. ad3 was added to 60 ng of the RsaI-digested cDNA.

[0095]

(1)-5. First Round of Subtraction

The tester and the driver produced in Sections 3 and 4 above were mixed, subjected to ethanol precipitation, and then dissolved in 1 μl of 1x PCR buffer. After a 5-minute incubation at 98°C, 1 μl of 1x PCR buffer + 1 M NaCl was added. After another 5 minutes of incubation at 98°C, the tester and the driver were hybridized at 68°C for 16 hours.

[0096]

With ad3S as the primer, the hybridized cDNA was amplified by incubating at 72°C for 5 minutes, and performing ten cycles of 30 seconds at 94°C, 30 seconds at 65°C, and 2 minutes at 72°C. Next, the amplified cDNA was digested with the Mung Bean Nuclease (Takara) and purified using a Qiaquick PCR Purification Kit. Then, it was amplified by incubating at 94°C for 2 minutes, and performing 13 PCR cycles of 30 seconds at 94°C, 30 seconds at 65°C, and 2 minutes at 72°C, and a final 2-minute incubation at 72°C.

[0097]

(1)-6. Normalization

1 μl of 2x PCR buffer was added to 8 ng of the cDNA amplified in the first round of subtraction. After incubating at 98°C for 5 minutes, 2 μl of 1x PCR buffer + 1 M NaCl was added. After another 5 minutes of incubation at 98°C, the cDNA was hybridized at 68°C for 16 hours.

[0098]

The hybridized cDNA was digested with RsaI, and purified using the Qiaquick PCR Purification Kit. Then, it was amplified with ad3S as the primer by incubating at 94°C for 2 minutes, and performing 11 PCR cycles of 30 seconds at 94°C, 30 seconds at 65°C, and 2 minutes at 72°C, and a final 2-minute incubation at 72°C. The PCR product was then digested with RsaI, followed by the addition of ad4.

[0099]

(1)-7. Second Round of Subtraction

20 ng of the cDNA to which ad4 was added in Section 6 above was used as the Tester and mixed with the Driver of 1-3 above, and the same subtraction procedure used in Section 1-5 above was performed. Finally, ad5 was added to the cDNA following RsaI digestion.

[0100]

(1)-8. Third Round of Subtraction

2 ng of the cDNA to which ad5 was added in section 7 above was used as the Tester and mixed with the Driver of section 3 above, and the same subtraction procedure used in section 5 above was carried out. Finally, ad13 was added to the RsaI-digested cDNA.

[0101]

(1)-9. Fourth Round of Subtraction

2 ng of the cDNA to which ad13 was added in section 8 above was used as the Tester and mixed with the Driver of section 3 above, and the same subtraction procedure used in section 5 above was carried out. The amplified cDNA was cloned into pCRII (Invitrogen), and its nucleotide sequence was analyzed using the ABI3100 sequence analyzer.

[0102]

[Example 2] Expression Analysis of the Lrp4 Gene

Next, an expression analysis of theLrp4 gene by *in situ* hybridization was carried out according to the following protocol.

[0103]

First, E12.5 mouse embryos were embedded in OCT, and fresh frozen sections of 16 μm thickness were prepared. After drying on a slide glass, the sections were fixed in 4% PFA at room temperature for 30 minutes. After washing with PBS, hybridization was carried

out at 65°C for 40 hours (1 μg/ml DIG-labeled RNA probe, 50% formamide, 5x SSC, 1% SDS, 50 μg/ml yeast RNA, 50 μg/ml Heparin). Subsequently, the sections were washed at 65°C (50% formamide, 5x SSC, 1% SDS) and then treated with RNase (5 μg/ml RNase) at room temperature for 5 minutes. After washing with 0.2x SSC at 65°C and washing with 1x TBST at room temperature, blocking was carried out (Blocking reagent: Roche). The sections were then reacted with alkaline phosphatase-labeled anti-DIG antibody (DAKO), washed (1x TBST, 2 mM Levamisole), and color developed using NBT/BCIP (DAKO) as the substrate.

[0104]

The expression analysis by *in situ* hybridization showed that Lrp4 mRNA is specifically expressed in the ventral midline region from the midbrain to the hindbrain and the spinal cord at the stage E12.5, which corresponds to the time of dopaminergic neuron development. Lrp4 demonstrates a similar expression pattern to Shh mRNA from the hindbrain to the spinal cord, and was clearly determined to be specific to the floor plate, which is the organizer region (Figs. 2 and 5). In the midbrain, Lrp4 expression was observed more centrally than the Shh mRNA expression zone (Figs. 3 and 5).

[0105]

The results of comparison with the neuron maturation marker NCAM mRNA show that Lrp4 mRNA-expressing cells were proliferative progenitor cells in the NCAM mRNA-negative ventricular zone (VZ). Moreover, when compared with the expression of the dopamine neuron marker, TH mRNA, their expression regions completely overlapped along the dorsal-ventral axis (Figs. 3 and 5), although expression of both TH mRNA and Lrp4 mRNA in the same cells was not observed since TH mRNA is only expressed in the mantle layer (ML). In general, neurons present in neural tubes are known to first proliferate in the VZ, stop cell division with the commencement of differentiation, and then mature after migrating to the outer ML. Thus, dopaminergic neuron progenitor cells are believed to proliferate in the VZ which lines the TH expression zone, and express TH mRNA after having migrated to the outside following cell division arrest. Namely, Lrp4 mRNA is believed to be specifically expressed in the midbrain in dopaminergic neuron progenitor cells (Figs. 4 and 6).

[0106]

[Example 3] Expression of Lrp4 in Dopaminergic Neurons Induced to Differentiate from ES Cells

[0107]

Next, whether Lrp4 is expressed in ES cells that have been induced to differentiate into dopaminergic neurons *in vitro*, was examined.

First, dopamine neurons were induced to differentiate from ES cells using the SDIA

method (Kawasaki *et al.* (2000) Neuron 28 (1): 31-40) (see the upper part of Fig. 7). Cells were recovered 4, 6, 8, 10, and 12 days after induction, and total RNA was recovered using the RNeasy Mini Kit (Qiagen) followed by RT-PCR. In RT-PCR, cDNA was initially synthesized for 1 μg of total RNA using the RNA PCR Kit (TaKaRa). PCR was then carried out in the following reaction system, using as a template cDNA equivalent to 10 ng, 1 ng, and 0.1 ng.

10x ExTaq	2 μl
2.5 mM dNTP	1.6 μl
ExTaq	0.1 μl
100 μM primers	0.2 μl each
cDNA	1 μl
Distilled water	14.9 μl

[0108]

After incubating for 2 minutes at 94°C, 35 PCR cycles of 30 seconds at 94°C, 30 seconds at 65°C, and 2 minutes at 72°C were carried out followed by incubating for 2 minutes at 72°C.

The sequences of the primers used are shown below.

[0109]

Lrp4: TAGTCTACCACTGCTCGACTGTAACG (SEQ ID NO: 15) /
CAGAGTGAACCCAGTGGACATATCTG (SEQ ID NO: 16)

TH: GTTCCCAAGGAAAGTGTCAGAGTTGG (SEQ ID NO: 17) /
GAAGCTGGAAAGCCTCCAGGTGTTCC (SEQ ID NO: 18)

DAT: CTCCGAGCAGACACCATGACCTTAGC (SEQ ID NO: 19) /
AGGAGTAGGGCTTGTCTCCCAACCTG (SEQ ID NO: 20)

[0110]

According to the results of expression analysis by RT-PCR, although Lrp4 is not expressed in ES cells (CCE) or stromal cells (PA6), expression was clearly induced starting on day 4 in the same manner as TH as a result of inducing differentiation (Fig. 8). Thus, Lrp4 is useful as markers not only when isolating dopaminergic neuron progenitor cells from the fetal midbrain, but also when isolating dopaminergic neuron progenitor cells that have been induced to differentiate from ES cells *in vitro*.

[0111]

[Example 4] Analysis of Lrp4 Protein Expression

The anti-Lrp4 antibody was produced by the protocol described below using the extracellular region encoding sequence in the Lrp4 gene, and expression analyses were performed using immunohistological staining.

[0112]

First, the extracellular region (161 to 502 amino acids) encoding sequence in the Lrp4 gene was introduced into 293E cells, and the extracellular region of the Lrp4 protein was expressed and collected. Hamsters were immunized with the collected protein, and lymphocytes were removed therefrom and fused with myeloma cells. The fused cells were transplanted into a cavity of mouse, and then ascites was obtained to purify anti-Lrp4 monoclonal antibody. E12.5 mouse embryos were then fixed with 4% PFA/PBS(-) at 4°C for two hours, then the solution was replaced with 20% sucrose/PBS(-) at 4°C overnight, and the embryos were embedded with OCT. 12-μm-thick sections were made, attached on a slide glass, dried at room temperature for 30 minutes, and wetted with PBS(-) again. The sections were blocked (10% normal donkey serum and 10% normal goat serum/Block Ace) at room temperature for 20 minutes, and then reacted with the anti-Lrp4 monoclonal antibody produced as described above (1/2-diluted culture supernatants, 10% normal donkey serum, 10% normal goat serum, and 2.5% Block Ace/PBS) and the anti-TH antibody (Chemicon, 0.7 μg/mL, 10% normal donkey serum, 10% normal goat serum, and 2.5% Block Ace/PBS) at room temperature for one hour, and further reacted at 4°C overnight. The sections were washed four times with 0.1% Triton X-100/PBS(-) at room temperature for 10 minutes. Cy3-labeled anti-hamster IgG antibody or the FITC-labeled anti-mouse IgG antibody (Jackson, 10 μg/mL, 10% normal donkey serum, 10% normal goat serum, and 2.5% Block Ace/PBS) was reacted with the sections at room temperature for one hour. Then, the sections were washed as described above, then washed with PBS(-) at room temperature for 10 minutes, and sealed.

[0113]

As a result of expression analysis by immunohistological staining using the produced anti-Lrp4 monoclonal antibody, expression of the Lrp4 protein was observed in the ventral midbrain at E12.5, the stage when the dopaminergic neuron developed (Fig. 8) as shown by expression analysis using *in situ* hybridization. Compared with expression of the TH protein, which was the dopaminergic neuron marker, the Lrp4 protein was expressed in the most ventral midbrain (VZ side) where the TH protein was expressed. Accordingly, it appeared that the Lrp4 protein is expressed in the dopaminergic neuron progenitor cells.

[0114]

Next, using the anti-Lrp4 monoclonal antibody, Lrp4-expressing cells were detected by flow cytometry.

[0115]

First, ES cells were induced to differentiate to dopaminergic neuron progenitor cells *in vitro* using the SDIA method. A cell population comprising the cells was dispersed using a cell dissociation buffer (Invitrogen), and stained with the anti-Lrp4 monoclonal antibody (1/2-

diluted culture supernatant, 1% fetal calf serum, and 1 mM EDTA/SDIA differentiation medium) at 4°C for 20 minutes without fixing and permeabilization. Subsequently, the cells were washed three times with 1% fetal calf serum and 1 mM EDTA/SDIA differentiation medium at 4°C for 3 minutes. The cells were stained with biotin-labeled anti-hamster IgG antibody (Jackson, 10 μg/mL, 1% fetal calf serum and 1 mM EDTA/SDIA differentiation medium) at 4°C for 20 minutes, and washed as described above. Then, the cells were stained with PE-labeled streptavidin (Pharmingen, 20 μg/mL, 1% fetal calf serum and 1 mM EDTA/SDIA differentiation medium) at 4°C for 20 minutes, and washed as described above. After staining, the Lrp4-expressing cells were detected by a flow cytometer.

[0116]

The results of detecting the Lrp4-expressing cells by flow cytometry using the produced anti-Lrp4 monoclonal antibody detected an Lrp4 protein-expressing population (Fig. 9). Since the Lrp4 protein-expressing cells could be detected without fixing and permeabilization, it appeared that living Lrp4 protein-expressing cells can be separated by using a flow cytometer equipped with a cell sorter. The Lrp4 protein is thought to be expressed in the dopaminergic neuron progenitor cells, and thus the Lrp4 antibody appeared to be useful for the separation of the dopaminergic neuron progenitor cells.

[0117]

[Example 5] Separation of Lrp4-expresseng Cells by Antibodies

Lrp4 protein-positive cells separated using the anti-Lrp4 antibody were characterized.

[0118]

First, a cell population comprising E12.5 mouse fetus ventral midbrain and dopaminergic neuron progenitor cells, induced *in vitro* to differentiate from ES cells by the SDIA method, was stained with the anti-Lrp4 antibody by the method described in Example 4. Lrp4-positive and -negative cells were separated using a cell sorter. Total RNA was collected from the cells immediately after separation using an RNeasy mini kit (Qiagen). Then, cDNA was synthesized, and amplified by the same method as described in Example 1 to use as a template in RT-PCR. The PCR was performed using cDNAs corresponding to the amplified cDNA equivalent to 4 ng, 0.4 ng, and 0.04 ng by the following reaction system.

10x ExTaq	1 μL
2.5 mM dNTP	0.8 μL
ExTaq	0.05 μL
100 μM primers	0.1 μL each
cDNA	1 μL
Distilled water	6.95 μL

[0119]

PCR was carried out under conditions of 94°C for 2 minutes, 26 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 2 minutes, and finally 72°C for 2 minutes.

The sequences of the primers used are shown below.

[0120]

Lrp4: TAGTCTACCACTGCTCGACTGTAACG (SEQ ID NO: 15) /
CAGAGTGAACCCAGTGGACATATCTG (SEQ ID NO: 16)

TH: GTTCCCAAGGAAAGTGTCAGAGTTGG (SEQ ID NO: 17) /
GAAGCTGGAAAGCCTCCAGGTGTTCC (SEQ ID NO: 18)

Nurr1: CACTCCTGTGTCTAGCTGCCAGATGC (SEQ ID NO: 21) /
AGTGCGAACACCGTAGTGCTGACAGG (SEQ ID NO: 22)

Nestin: GATGAAGAAGAAGGAGCAGAGTCAGG (SEQ ID NO: 23) /
ATTCACTTGCTCTGACTCCAGGTTGG (SEQ ID NO:24)

MAP2: CCATGATCTTTCCCCTCTGGCTTCTG (SEQ ID NO: 25) /
TTTGGCTGGAAAGGGTGACTCTGAGG (SEQ ID NO: 26)

[0121]

As expected, the results of expression analysis by the RT-PCR indicated expression of the proliferative progenitor cell marker Nestin. It was also revealed that cells expressing MAP2, which was the postmitotic neuron marker, were included in the Lrp4 protein-positive cell population (Fig. 10). Therefore, it was found that Lrp4 protein expression is maintained after stopping mRNA expression, and that Lrp4 protein is useful as a marker for separating not only dopamine neuron proliferative progenitor cells but also postmitotic dopamine neurons (Fig. 11). Furthermore, since Nurr1 and TH, which are markers for postmitotic dopamine neurons, were expressed at higher levels compared to the Lrp4-negative cell population, Lrp4-positive cells were confirmed to be dopamine neuron lineage progenitor cells (Fig. 10).

[0122]

Next, the present inventors analyzed the ratio of proliferative progenitor cells to postmitotic progenitor cells in the Lrp4 protein-positive cell population separated by the anti-Lrp4 antibody.

[0123]

The separated cells were seeded on a glass slide coated with poly-L-ornithine (Sigma, 0.002% in PBS), laminin (Invitrogen, 5 μg/mL in PBS), and fibronectin (Sigma, 5 μg/mL in PBS), and incubated in N2 (Invitrogen, 1x), B27 (Invitrogen 1x), ascorbic acid (Sigma, 200 μM), and BDNF (Invitrogen, 20 ng/mL)/SDIA differentiation medium at 37°C for 40 minutes to adhere thereon. The adherent cells were fixed in 4% PFA/PBS at 4°C for 20 minutes, and washed twice with PBS at 4°C for 10 minutes. Permeabilization with 0.3% Triton X-100/PBS was performed at room temperature for 15 minutes, and blocking with 10% normal

donkey serum/Block Ace was performed at room temperature for 20 minutes. Then, the cells were reacted with anti-Nestin antibody (Chemicon, 2 μg/mL, 10% normal donkey serum, 2.5% Block Ace, 0.1% Triton X-100/PBS) or anti-βIII-tubulin antibody (BABCO, 1/2,000, 0.5 μg/mL, 10% normal donkey serum, 2.5% Block Ace, and 0.1% Triton X-100/PBS) at room temperature for one hour, and subsequently at 4°C overnight. On the next day, the cells were washed three times with 0.1% Triton X-100/PBS at room temperature for 5 minutes, and reacted with the FITC-labeled anti-mouse IgG antibody or the Cy5-labeled anti-rabbit IgG antibody (both from Jackson, 10 μg/mL, 10% normal donkey serum, 2.5% Block Ace, and 0.1% Triton X-100/PBS) at room temperature for 30 minutes. Subsequently, the cells were washed as described above, then washed with PBS at room temperature for 5 minutes, and sealed for observation.

[0124]

Also, the separated cells were similarly seeded on a glass slide, and cultured in the above-described medium supplemented with BrdU (Roche, 5-Bromo-2'-deoxy-uridine Labeling and Detection kit II, 1x) at 37°C for 18 hours. Then, the above-described procedures were similarly carried out until the blocking step. The cells were reacted in 2 N HCl at 37°C for 20 minutes, washed three times with PBS, and then reacted with anti-BrdU antibody and DNase (Roche, 5-Bromo-2'-deoxy-uridine Labeling and Detection kit II, 1x conc. in incubation buffer) at 37°C for 30 minutes. Furthermore, the cells were reacted with anti-BrdU antibody (Sigma, 44 μg/mL, 10% normal donkey serum, 2.5% Block Ace, and 0.1% Triton X-100/PBS) at room temperature for one hour, and subsequently at 4°C overnight. The next day, the cells were washed three times with 0.1% Triton X-100/PBS at room temperature for 5 minutes, and then reacted with the FITC-labeled anti-mouse IgG antibody (Jackson, 10 μg/mL, 10% normal donkey serum, 2.5% Block Ace, and 0.1% Triton X-100/PBS) at room temperature for 30 minutes. Subsequently, the cells were washed, and sealed for observation as described above.

[0125]

As a result of the marker staining, it was revealed that a majority of the Lrp4-positive cells are Nestin-positive proliferative progenitor cells and that a part thereof is positive for the postmitotic marker III-tubulin (Fig. 12). In addition, the separated cells were confirmed to frequently incorporate BrdU, and to actually proliferate *in vitro* (Fig. 13).

[0126]

Next, the present inventors confirmed that the separated Lrp4-positive cells differentiate into the dopaminergic neurons.

[0127]

The separated cells were seeded on a glass slide coated with poly-L-ornithine (Sigma,

0.002% in PBS), laminin (Invitrogen, 5 μg/mL in PBS), and fibronectin (Sigma, 5 μg/mL in PBS), and incubated in N2 (Invitrogen, 1x), B27 (Invitrogen 1x), ascorbic acid (Sigma, 200 μM), BDNF (Invitrogen, 20 ng/mL), and bFGF (R&D, 10 ng/ml)/SDIA differentiation medium at 37°C for 24 hours. The cells were then further cultured in the above medium without bFGF for another six days. The cultured cells were fixed in 4% PFA/PBS at 4°C for 20 minutes, and washed twice with PBS at 4°C for 10 minutes. Permeabilization with 0.3% Triton X-100/PBS was performed at room temperature for 15 minutes, and blocking with 10% normal donkey serum/Block Ace was performed at room temperature for 20 minutes. Then, the cells were reacted with anti-TH antibody (Chemicon, 0.3 μg/mL, 10% normal donkey serum, 2.5% Block Ace, 0.1% Triton X-100/PBS) or anti-βIII-tubulin antibody (BABCO, 1/2,000, 0.5 μg/mL, 10% normal donkey serum, 2.5% Block Ace, and 0.1% Triton X-100/PBS) at room temperature for one hour, and subsequently at 4°C overnight. On the next day, the cells were washed three times with 0.1% Triton X-100/PBS at room temperature for 5 minutes, and reacted with the FITC-labeled anti-mouse IgG antibody or the Cy5-labeled anti-rabbit IgG antibody (both from Jackson, 10 μg/mL, 10% normal donkey serum, 2.5% Block Ace, and 0.1% Triton X-100/PBS) at room temperature for 30 minutes. Subsequently, the cells were washed as described above, then washed with PBS at room temperature for 5 minutes, and sealed for observation.

[0128]

As a result of culturing the separated cells *in vitro*, it was obvious that more TH protein-positive dopaminergic neurons were induced than with the unseparated control cells. Therefore, it was revealed that the Lrp4-positive cells are certainly dopamine neuron lineage progenitor cells and can mature *in vitro* (Fig. 14).

[Industrial Applicability]

[0129]

The present invention identified Lrp4 as a gene expressed specifically and transiently in dopaminergic neuron proliferative progenitor cells prior to cell division arrest. As a result of examining Lrp4 expression in more detail, it was confirmed that Lrp4 mRNA and the Lrp4 protein were expressed specifically in dopaminergic neuron proliferative progenitor cells and in dopaminergic neuron progenitor cells, including cells prior to and after cell division arrest, respectively. Thus, by using the expression of Lrp4 mRNA or polypeptide in the cells as an index, it became possible in terms of safety, survival rate, and network formation to select dopaminergic neuron lineage cells suitable for transplant therapy for neurodegenerative diseases including Parkinson's disease. When the cells are obtained using Lrp4 as a marker, as in the present invention, the cells can be easily differentiated into a suitable state *in vitro* even when the therapy requires mature cells. Moreover, dopaminergic neuron progenitor

cells obtained by the methods of the present invention can also be used to isolate genes specifically expressed in these cells. The cells are also thought to be useful in developing pharmaceuticals for neurodegenerative diseases such as Parkinson's disease. Since dopaminergic neuron proliferative progenitor cells prior to cell division arrest obtained using Lrp4 mRNA as a marker are involved in early neuron formation, they are useful in elucidating the neuron maturation process, namely, for identifying various factors involved in the maturation process. Elucidation of these factors is expected to contribute greatly to the treatment of neurodegenerative diseases. Moreover, maturation of these cells can be used as an index for screening substances that may regulate (inhibit or promote) the maturation process.

[Brief Description of the Drawings]

[0130]

[Fig. 1] Fig. 1 schematically shows the structure of Lrp4. TM: transmembrane domain, FRI: frizzeled domain, LDLa: LDL receptor domain, SR: scavenger receptor domain, Protease: serine protease domain.

[Fig. 2] Fig. 2 is a set of photographs showing the results of Lrp4 and Shh mRNA expression analysis in E12.5 mouse ventral hindbrain region and spinal cord by *in situ* hybridization.

[Fig. 3] Fig. 3 is a set of photographs showing the results of Lrp4, Shh, tyrosine hydroxylase (TH), and NCAM mRNA expression analysis in E12.5 mouse ventral midbrain region by *in situ* hybridization.

[Fig. 4] Fig. 4 is a schematic diagram of the Lrp4 expression pattern in the midbrain, and photographs indicating the expression of mRNAs of Lrp4, tyrosine hydroxylase (TH), Sim-1, and NCAM in the ventral midbrain of E12.5 mice analyzed by *in situ* hybridization. VZ: ventricular zone; and ML: mantle layer.

[Fig. 5] Fig. 5 is a set of photographs showing the results of Lrp4 mRNA expression analysis in the E12.5 mouse central nervous system by *in situ* hybridization. A: sagittal cross-section, B: enlarged photograph of the area inside the box in A, C: cross-section at the location of the red line in A, D: Expression of Lrp4, Shh, and tyrosine hydroxylase (TH) mRNA in the E12.5 mouse midbrain ventral region.

[Fig. 6] Fig. 6 schematically shows the timing of expression of Lrp4, NCAM, TH, and DAT mRNAs from the generation to maturation of dopaminergic neurons.

[Fig. 7] Fig. 7 shows an expression of Lrp4 in *in vitro* dopaminergic neuron differentiation lineage from ES cells. The top panel consisting of a drawing and photograph schematically shows the inhibition of differentiation of ES cells into dopaminergic neurons. The bottom photograph shows the results of investigating the expression of Lrp4 mRNA in

dopaminergic neurons differentiated from ES cells over time, using the SDIA method by RT-PCR.

[Fig. 8] Fig. 8 is a photograph showing Lrp4 protein expression in the E12.5 mouse midbrain.

[Fig. 9] Fig. 9 shows Lrp4 protein expression on the SDIA-differentiated cell surface analyzed by FACS using the anti-Lrp4 antibody.

[Fig. 10] Fig. 10 is a set of photographs showing the expression of various dopaminergic neuron markers in Lrp4-positive cells analyzed by RT-PCR.

[Fig. 11] Fig. 11 schematically shows the expression periods of Lrp4 mRNA and protein, and TH mRNA from the development to maturation of dopaminergic neurons. This shows that both dopaminergic neuron proliferative progenitor cells and postmitotic dopaminergic neuron progenitor cells are present in Lrp4-expressing cells.

[Fig. 12] Fig. 12 is a set of photographs showing the results of examining the differentiation stages of Lrp4-positive cells.

[Fig. 13] Fig. 13 is a set of photographs showing the *in vitro* proliferation of Lrp4-positive cells.

[Fig. 14] Fig. 14 is a set of photographs showing that Lrp4-positive cells differentiate into dopaminergic neurons.

[Fig. 15] Fig. 15 schematically shows the separation and application of dopaminergic neuron progenitor cells using anti-Lrp4 antibody.

[Sequence Listing]

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<130> E1-A0404

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<170> PatentIn version 3.1

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Ile Thr His Ser Gln Cys Gln Ile Leu Pro Tyr His Ser Thr Leu Ala
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725 730 735

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Gln Glu Leu Leu Val Tyr Arg His Ser Cys Pro Ser Arg Ser Glu Ile
835 840 845

Ser Leu Leu Cys Ser Lys Gln Asp Cys Gly Arg Arg Pro Ala Ala Arg
850 855 860

Met Asn Lys Arg Ile Leu Gly Gly Arg Thr Ser Arg Pro Gly Arg Trp
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Pro Trp Gln Cys Ser Leu Gln Ser Glu Pro Ser Gly His Ile Cys Gly
885 890 895

Cys Val Leu Ile Ala Lys Lys Trp Val Leu Thr Val Ala His Cys Phe
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Glu Gly Arg Glu Asp Ala Asp Val Trp Lys Val Val Phe Gly Ile Asn
915 920 925

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930 935 940

Ile Leu Leu His Pro Arg Tyr Ser Arg Ala Val Val Asp Tyr Asp Ile
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Ser Val Val Glu Leu Ser Asp Asp Ile Asn Glu Thr Ser Tyr Val Arg
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Pro Val Cys Leu Pro Ser Pro Glu Glu Tyr Leu Glu Pro Asp Thr Tyr
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Ala Glu His Trp Tyr Cys Asp Gly Asp Thr Asp Cys Lys Asp Gly Ser
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Leu Ser Asp Asp Val Ile Pro Leu Ala Asp Val Arg Ser Ala Val Ala
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Thr Asp Thr Ile Ser Arg Ala Lys Trp Asp Gly Thr Gly Gln Glu Val

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770 775 780

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785 790 795 800

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Phe Leu Ile Phe Ala Arg Arg Ile Asp Ile Arg Met Val Ser Leu Asp
995 1000 1005

Ile Pro Tyr Phe Ala Asp Val Val Val Pro Ile Asn Ile Thr Met
1010 1015 1020

Lys Asn Thr Ile Ala Ile Gly Val Asp Pro Gln Glu Gly Lys Val

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Tyr Trp Ser Asp Ser Thr Leu His Arg Ile Ser Arg Ala Asn Leu
1040 1045 1050

Asp Gly Ser Gln His Glu Asp Ile Ile Thr Thr Gly Leu Gln Thr
1055 1060 1065

Thr Asp Gly Leu Ala Val Asp Ala Ile Gly Arg Lys Val Tyr Trp
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Thr Asp Thr Gly Thr Asn Arg Ile Glu Val Gly Asn Leu Asp Gly
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Ser Met Arg Lys Val Leu Val Trp Gln Asn Leu Asp Ser Pro Arg
1100 1105 1110

Ala Ile Val Leu Tyr His Glu Met Gly Phe Met Tyr Trp Thr Asp
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Trp Gly Glu Asn Ala Lys Leu Glu Arg Ser Gly Met Asp Gly Ser
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Asp Arg Ala Val Leu Ile Asn Asn Asn Leu Gly Trp Pro Asn Gly
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Leu Thr Val Asp Lys Ala Ser Ser Gln Leu Leu Trp Ala Asp Ala

1160 1165 1170

His Thr Glu Arg Ile Glu Ala Ala Asp Leu Asn Gly Ala Asn Arg
1175 1180 1185

His Thr Leu Val Ser Pro Val Gln His Pro Tyr Gly Leu Thr Leu
1190 1195 1200

Leu Asp Ser Tyr Ile Tyr Trp Thr Asp Trp Gln Thr Arg Ser Ile
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His Arg Ala Asp Lys Gly Thr Gly Ser Asn Val Ile Leu Val Arg
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Ser Asn Leu Pro Gly Leu Met Asp Met Gln Ala Val Asp Arg Ala
1235 1240 1245

Gln Pro Leu Gly Phe Asn Lys Cys Gly Ser Arg Asn Gly Gly Cys
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Ser His Leu Cys Leu Pro Arg Pro Ser Gly Phe Ser Cys Ala Cys
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Pro Thr Gly Ile Gln Leu Lys Gly Asp Gly Lys Thr Cys Asp Pro
1280 1285 1290

Ser Pro Glu Thr Tyr Leu Leu Phe Ser Ser Arg Gly Ser Ile Arg

1295 1300 1305

Arg Ile Ser Leu Asp Thr Ser Asp His Thr Asp Val His Val Pro
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Val Pro Glu Leu Asn Asn Val Ile Ser Leu Asp Tyr Asp Ser Val
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Asp Gly Lys Val Tyr Tyr Thr Asp Val Phe Leu Asp Val Ile Arg
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Tyr Ala Ile Gly Gly Leu Leu Ser Ile Leu Leu Ile Leu Val Val
1670 1675 1680

Ile Ala Ala Leu Met Leu Tyr Arg His Lys Lys Ser Lys Phe Thr
1685 1690 1695

Asp Pro Gly Met Gly Asn Leu Thr Tyr Ser Asn Pro Ser Tyr Arg

1700 1705 1710

Thr Ser Thr Gln Glu Val Lys Ile Glu Ala Ile Pro Lys Pro Ala
1715 1720 1725

Met Tyr Asn Gln Leu Cys Tyr Lys Lys Glu Gly Gly Pro Asp His
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Asn Tyr Thr Lys Glu Lys Ile Lys Ile Val Glu Gly Ile Cys Leu
1745 1750 1755

Leu Ser Gly Asp Asp Ala Glu Trp Asp Asp Leu Lys Gln Leu Arg
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Ser Ser Arg Gly Gly Leu Leu Arg Asp His Val Cys Met Lys Thr
1775 1780 1785

Asp Thr Val Ser Ile Gln Ala Ser Ser Gly Ser Leu Asp Asp Thr
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Glu Thr Glu Gln Leu Leu Gln Glu Glu Gln Ser Glu Cys Ser Ser
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1835 1840 1845

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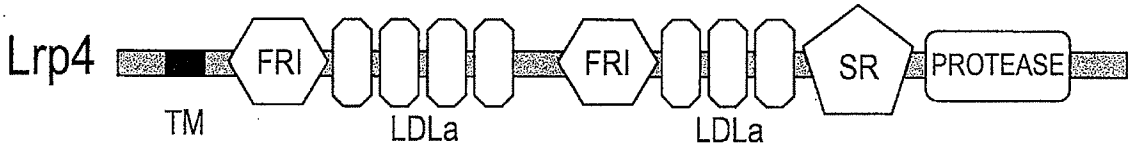
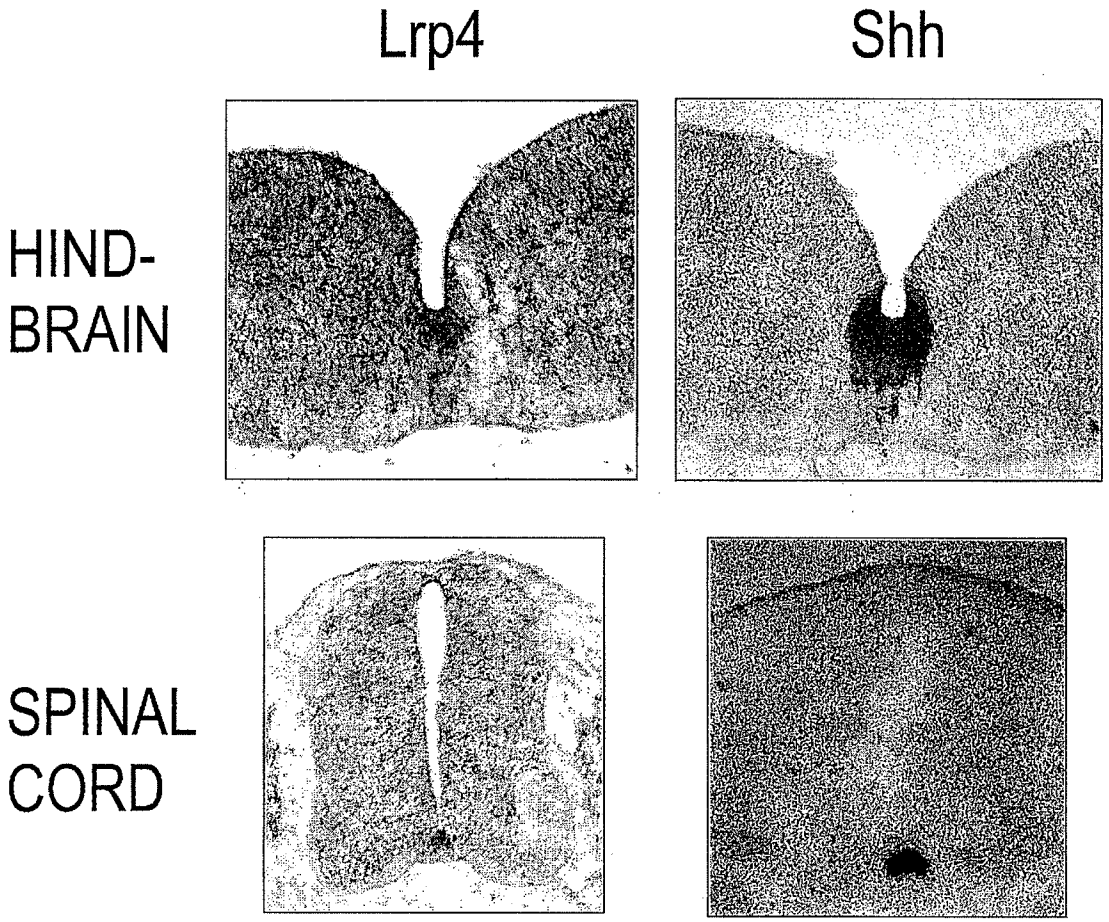
[Document Name] Drawings

[Fig. 1]



[Fig. 2]



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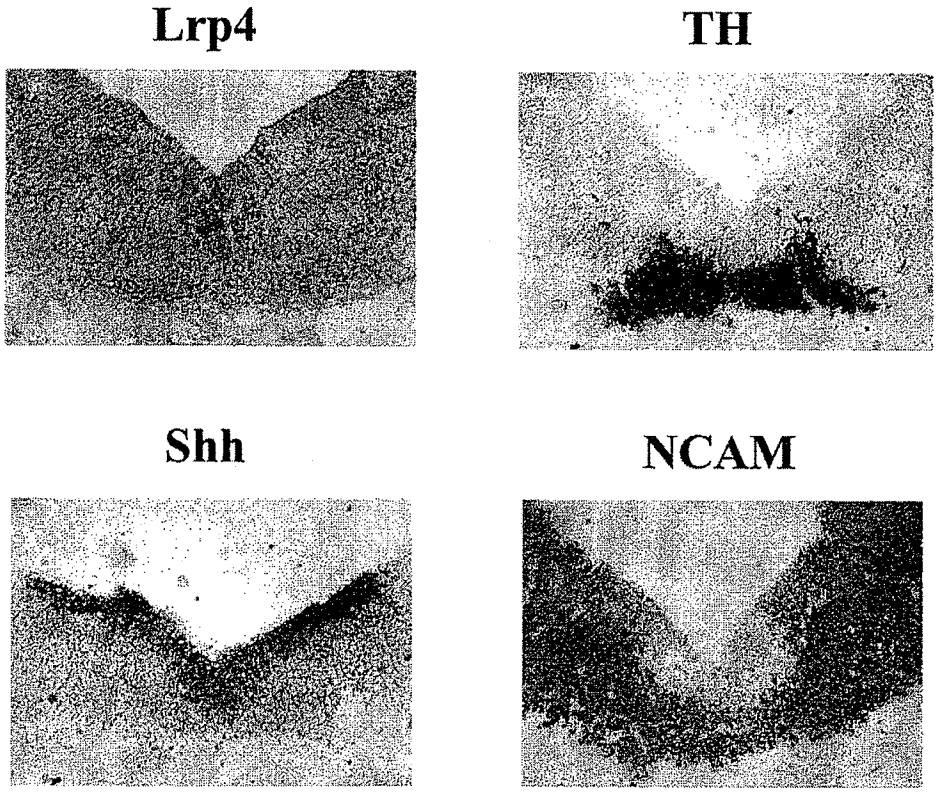
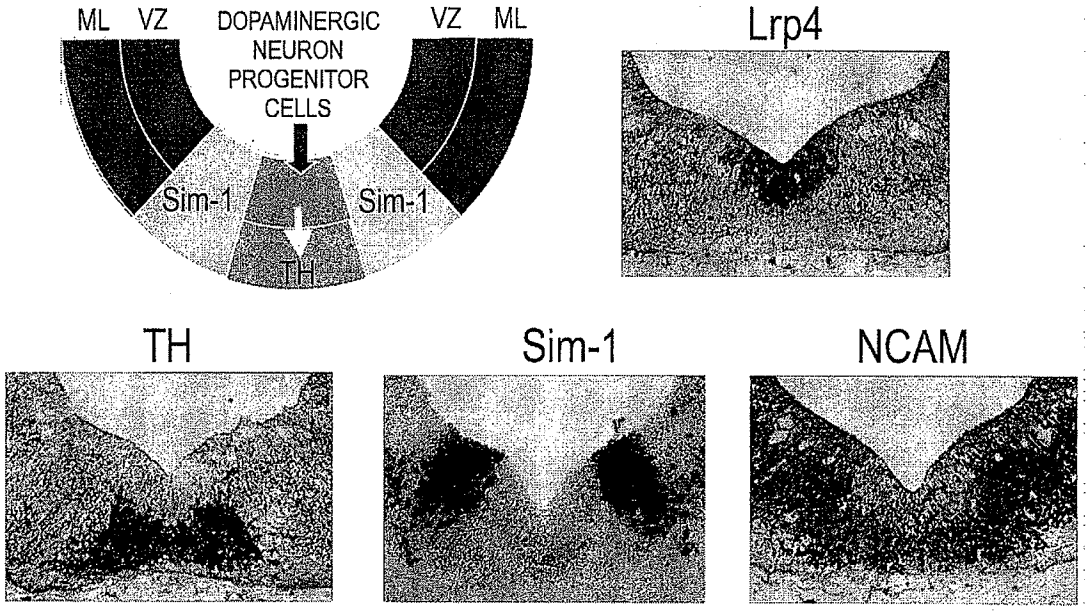
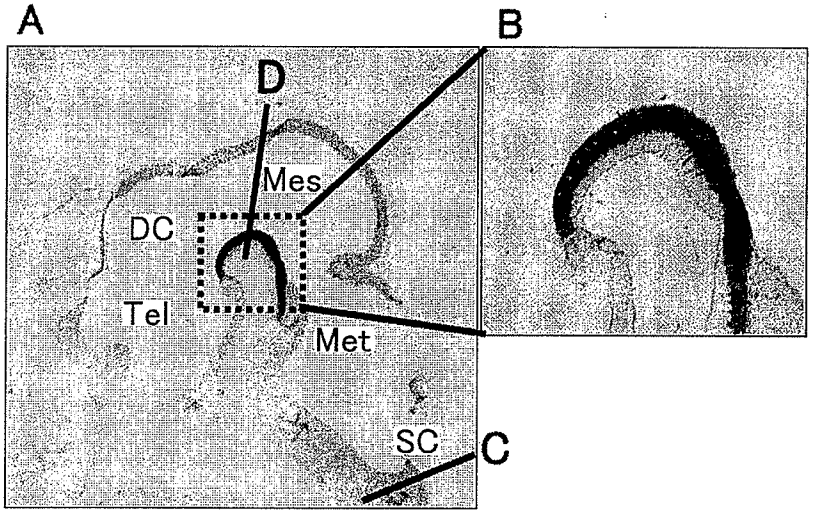

Lrp4
TH
Shh
NCAM

[Fig. 4]


ML
VZ
DOPAMINERGIC NEURON PROGENITOR CELLS
VZ
ML
Sim-1
Sim-1
TH
Lrp4
TH
Sim-1
NCAM

[Fig. 5]



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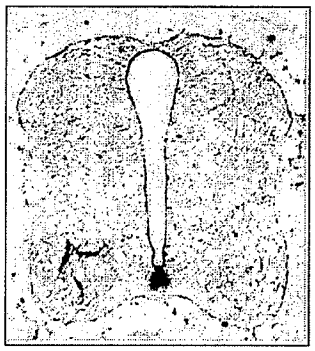
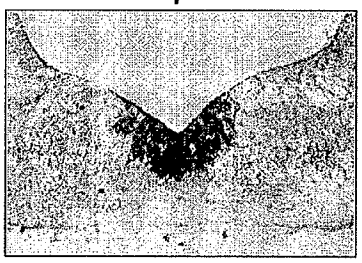
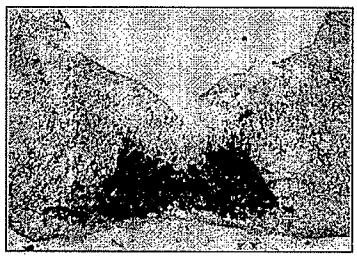


D. MIDBRAIN

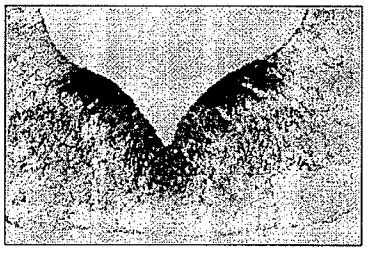
Lrp4



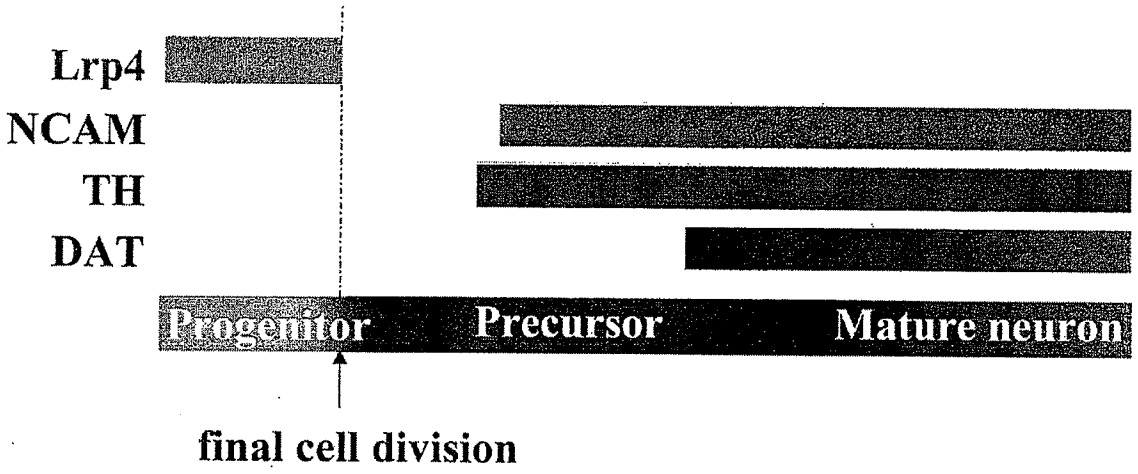
TH



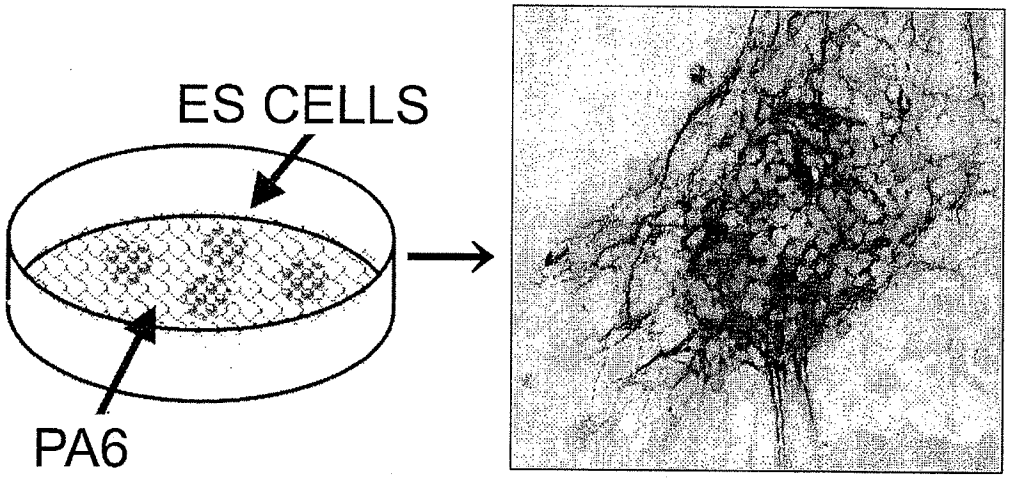
Shh

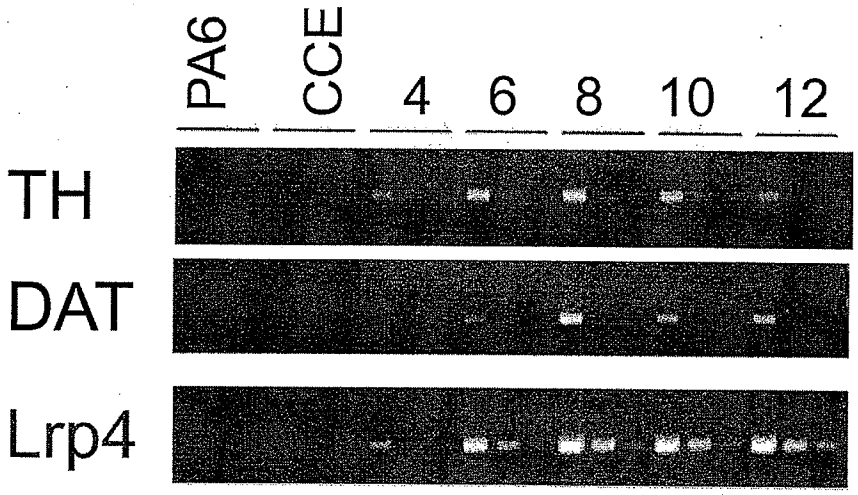


[Fig. 6]

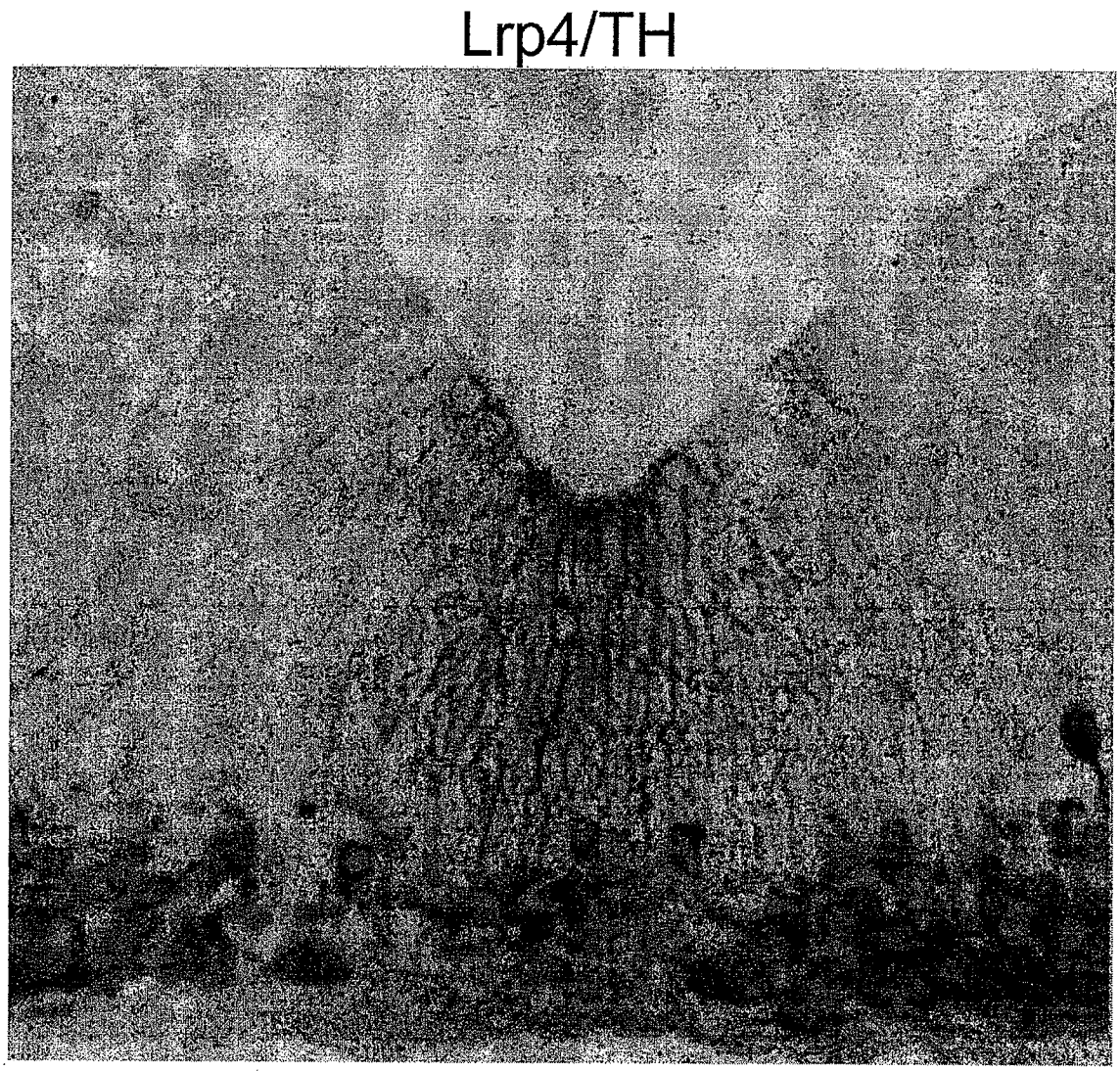

Lrp4
NCAM
TH
DAT
Progenitor
Precursor
Mature neuron
final cell division

[Fig. 7]

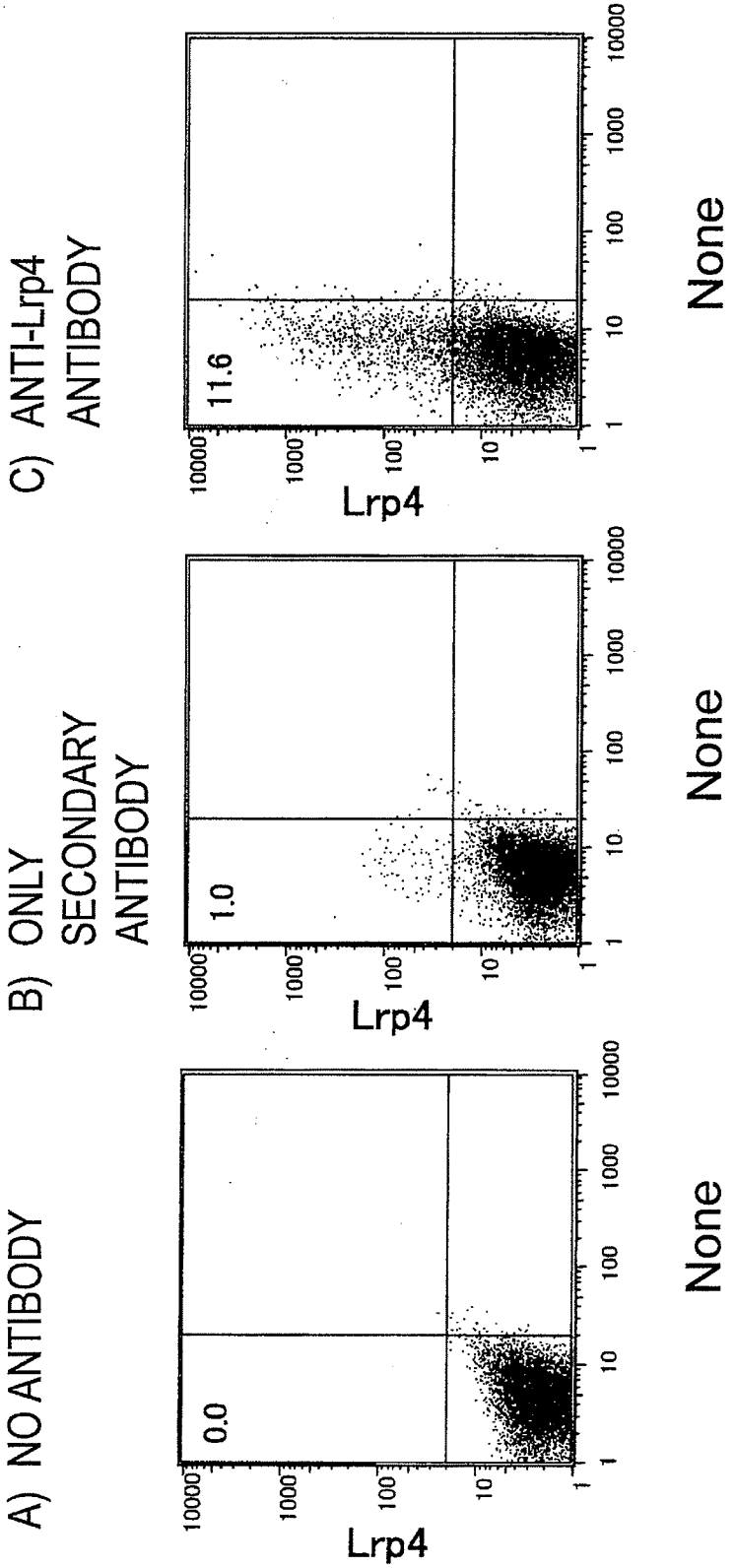

ES CELLS
PA6
TH/MAP2


PA6
CCE
4
6
8
10
12
TH
DAT
Lrp4

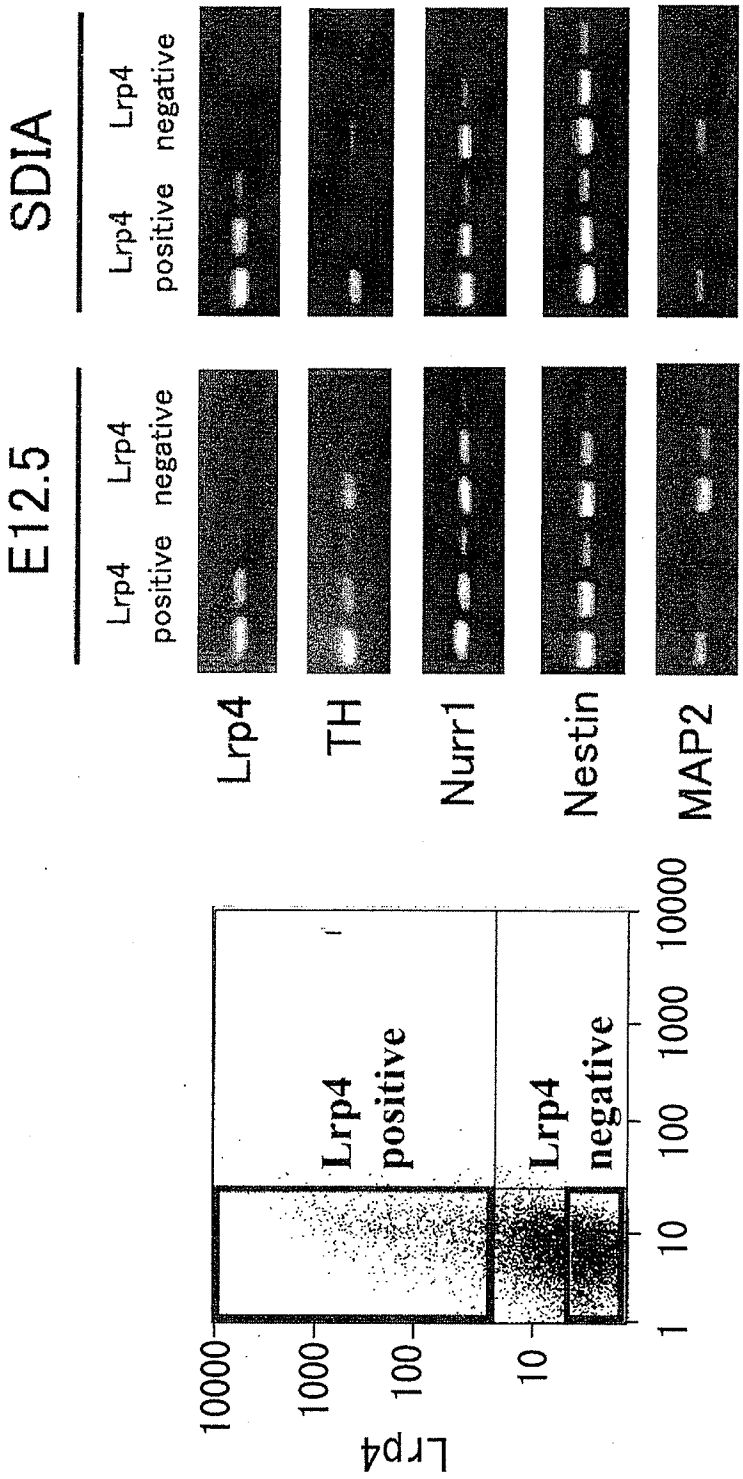
[Fig. 8]



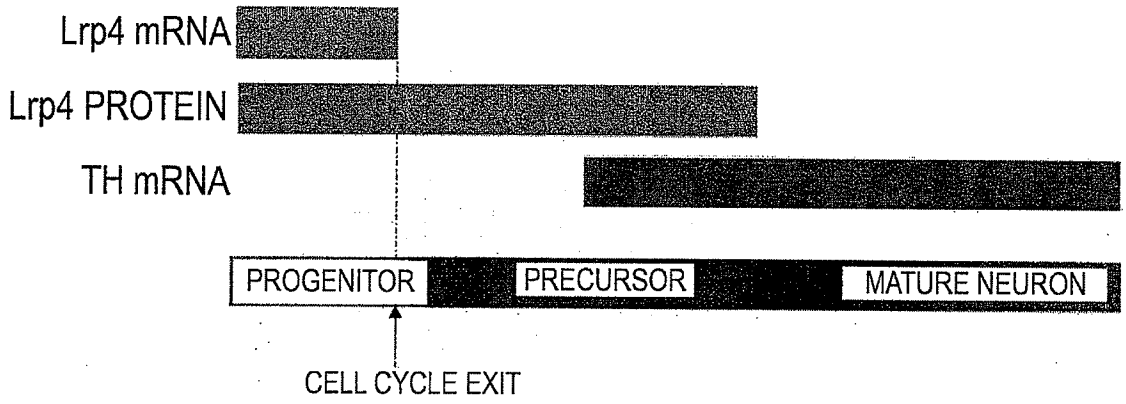
[Fig. 9]


A) NO ANTIBODY
B) ONLY SECONDARY ANTIBODY
C) ANTI-Lrp4 ANTIBODY
0.0
1.0
11.6
Lrp4
None
None
None
1
10
100
1000
10000

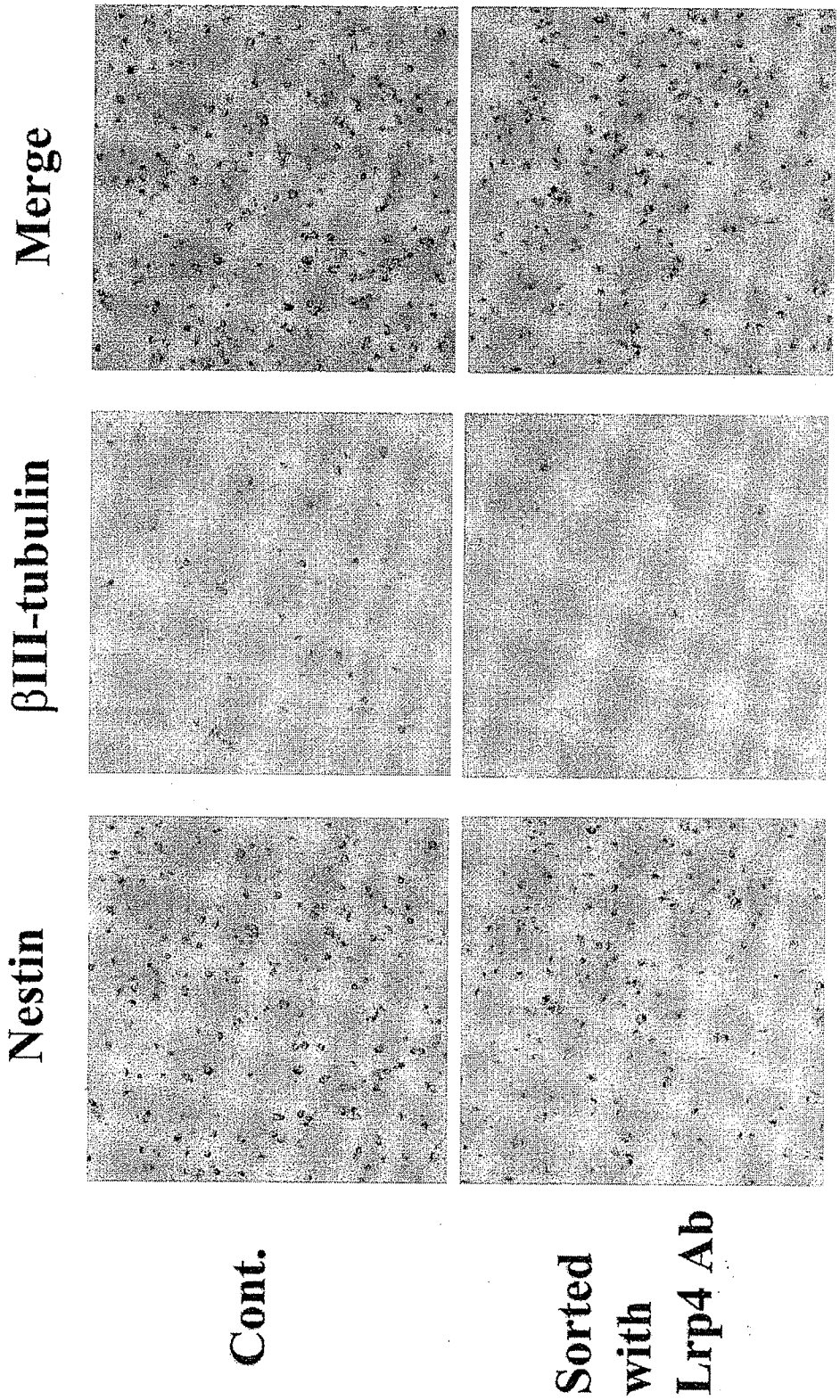
[Fig. 10]


E12.5
Lrp4 positive
Lrp4 negative
SDIA
Lrp4 positive
Lrp4 negative
Lrp4
TH
Nurr1
Nestin
MAP2
Lrp4
10000
1000
100
10
1
10
100
1000
10000
Lrp4 positive
Lrp4 negative

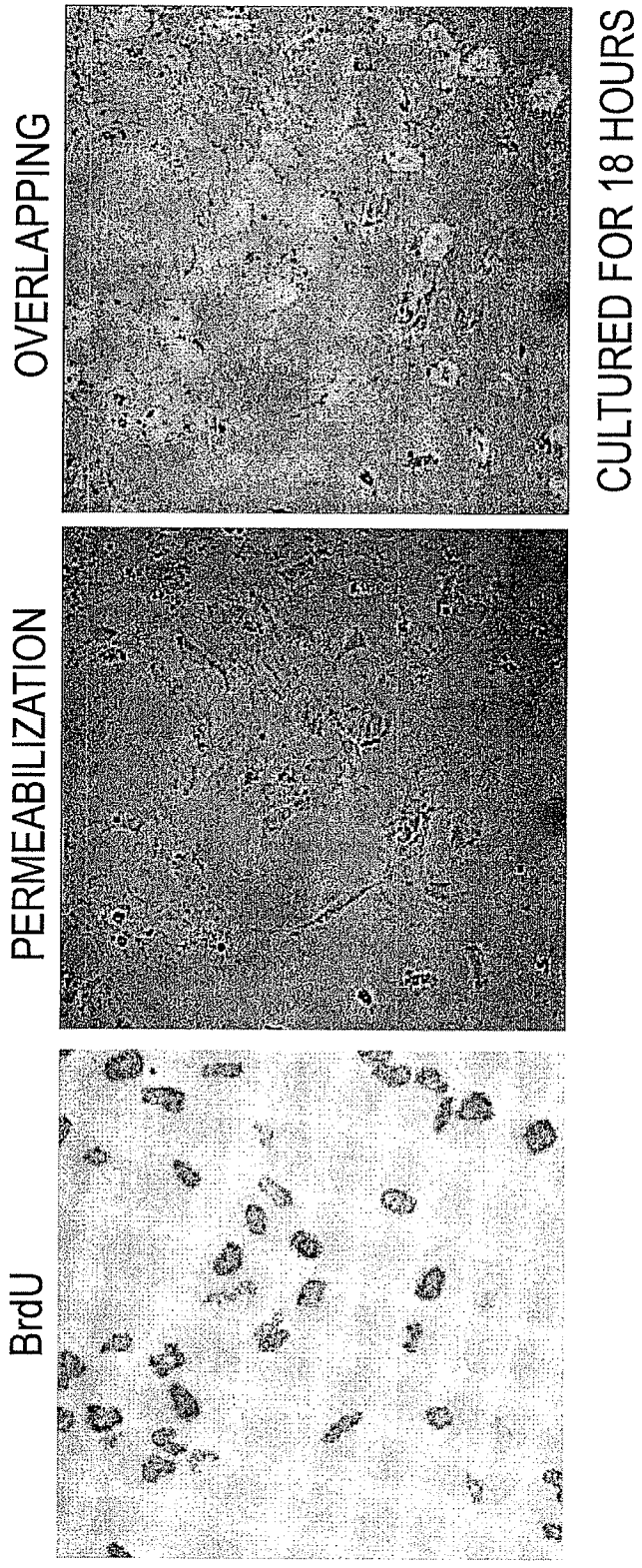
[Fig. 11]


Lrp4 mRNA
Lrp4 PROTEIN
TH mRNA
PROGENITOR
PRECURSOR
MATURE NEURON
CELL CYCLE EXIT

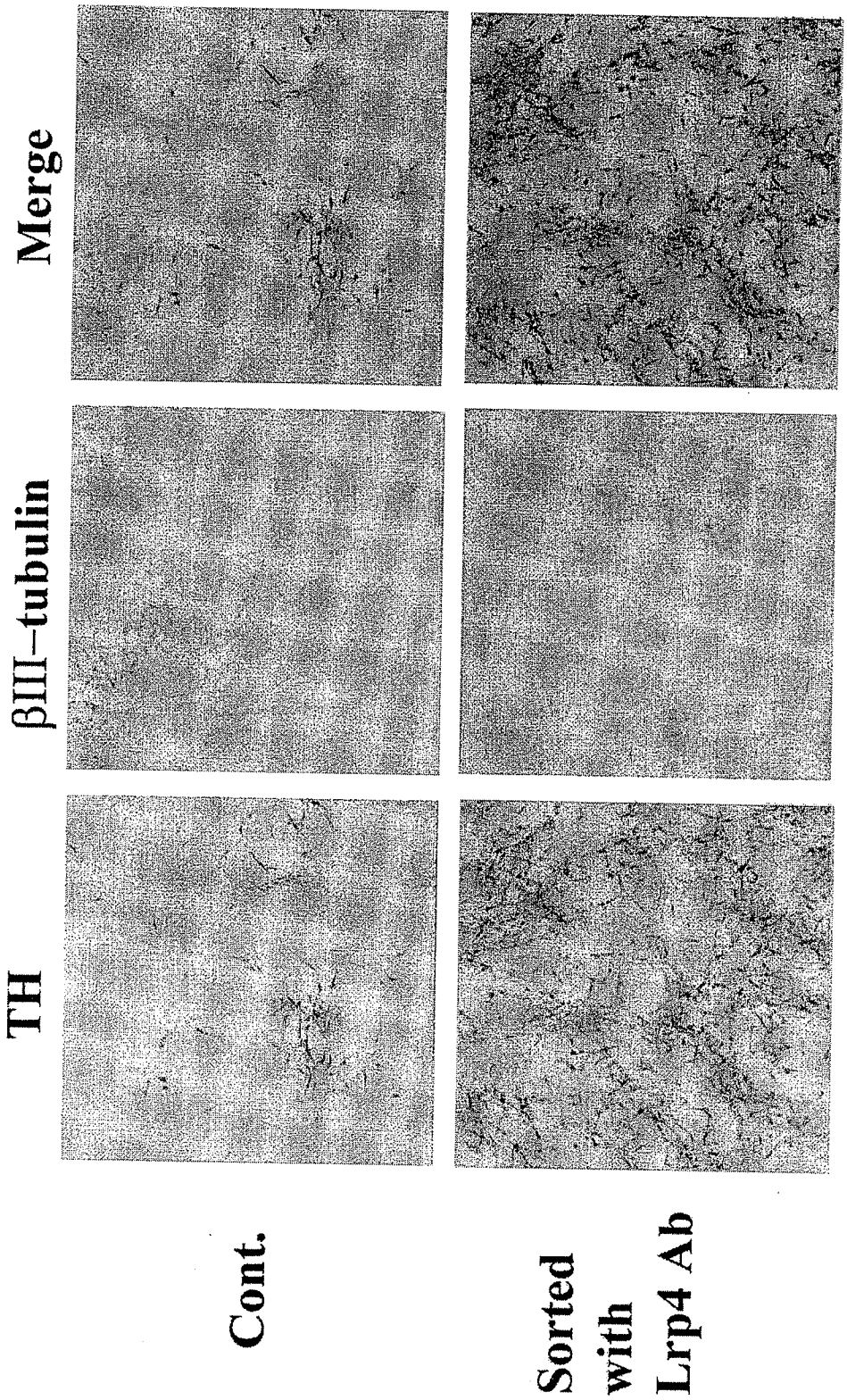
[Fig. 12]



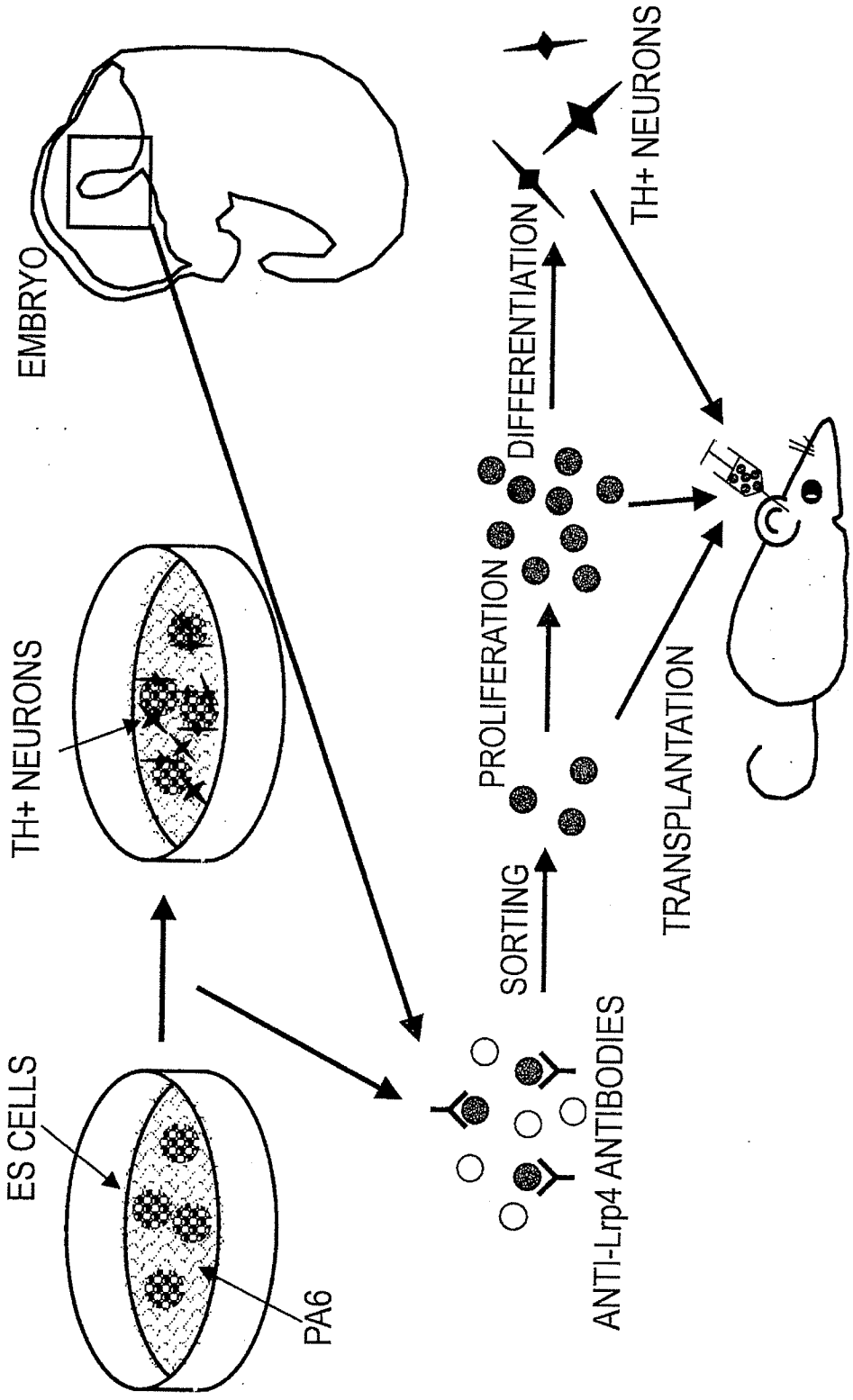
[Fig. 13]



[Fig. 14]



[Fig. 15]


EMBRYO
TH+ NEURONS
ES CELLS
PA6
ANTI-Lrp4 ANTIBODIES
SORTING
PROLIFERATION
DIFFERENTIATION
TH+ NEURONS
TRANSPLANTATION

[Document Name] Abstract

[Abstract]

[Problems to Be Solved] In neuron cell transplantation therapy, when considering safety standpoint, it is believed that cell populations that comprise only the cell type of interest is preferred, and when considering the risk of tumorigenesis, it is believed that postmitotic neuron cells are preferred. Moreover, when considering the survival of cells at their transplantation site, and their ability to properly form a network, it is expected that therapeutic effects can be further improved by progenitor cells at as early a stage as possible.

[Means for Solving the Problems] Lrp4, encoding a transmembrane protein, was identified as dopaminergic neuron progenitor cell-specific gene. It was confirmed that Lrp4 mRNA and protein specifically express dopaminergic neuron proliferative progenitor cells and dopaminergic neuron progenitor cells prior to and after cell division arrest, respectively. Thus, the present invention relates to polynucleotide probes and antibodies for detecting Lrp4/Corin dopaminergic neuron progenitor cell markers, which enable the efficient isolation of dopaminergic neuron progenitor cells; and methods for selecting the progenitor cells by the use thereof. Using Lrp4 expression on cells as an index, cells suitable for transplantation therapy of neurodegenerative diseases including Parkinson's disease can be selected in terms of safety, survival rate, and network formation ability.

[Selected Drawings] None